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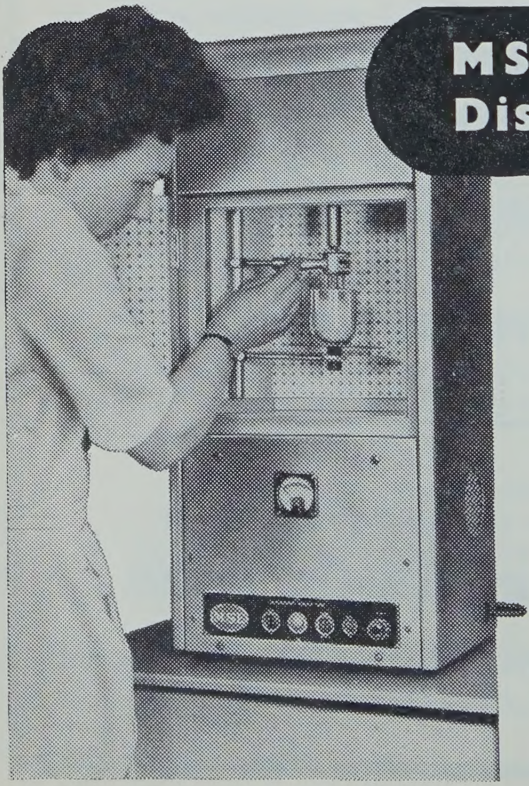

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Inhibition of Rooting of Cuttings by Gibberellic Acid

BY

P. W. BRIAN, H. G. HEMMING, and D. LOWE

(*Akers Research Laboratories, Imperial Chemical Industries Ltd., Welwyn*)

With one Figure in the Text

ABSTRACT

Gibberellic acid inhibited rooting but increased extension of pea and bean stem cuttings. Indolylacetic acid usually had a contrary effect both on extension and on root initiation. It was found possible to separate the effects of gibberellic acid on extension and rooting. Small doses of gibberellic acid applied to the bases of cuttings reduced rooting without increasing extension. Small apical doses increased extension without reducing rooting. Disbudding cuttings prevented extension, but gibberellic acid inhibited rooting of disbudded cuttings as strongly as that of normal cuttings. It has thus been found necessary to discard an earlier hypothesis that inhibition of rooting by gibberellic acid was a consequence of a diversion of essential metabolites to the extending apical regions of the cutting. The antagonism between gibberellic acid and indolylacetic acid was non-competitive. Gibberellin A_1, known to occur naturally in *Phaseolus*, was as active a rooting inhibitor as gibberellic acid. It is now believed that inhibition of rooting of cuttings by gibberellins is a direct local effect, preventing those early cell divisions involved in transformation of mature stem tissues to a meristematic condition.

INTRODUCTION

GIBBERELLINS have been shown to inhibit rooting of stem-cuttings of *Pisum* (Brian, Hemming, and Radley, 1955; Brian, 1957; Kato, 1958), *Salix* (Gundersen, 1958), and *Citrus* (Chaylakhian and Nekrassova, 1958). It also inhibits formation of adventitious roots on *Begonia* leaves (Schraudolf and Reinert, 1959) and on stems of intact Bryophyllum (Viana, 1958). It thus appears that, in their effects on the rooting of cuttings, the gibberellins are clearly distinguished from the auxins, which usually stimulate rooting. Gibberellins cause stem cuttings to elongate, and it has been suggested (Brian, 1957) that the failure to develop adventitious roots at the base of a cutting after gibberellin treatment may be due to diversion of essential metabolites to the extending apical regions of the cutting. It seemed significant, in this connexion, that indolylacetic acid, which stimulated root initiation, tended to suppress elongation of the cutting. This concept of competition between stem growth and root initiation had previously been used by Galston (1948) to explain observations on root initiation in excised *Asparagus* stem-tips.

We present below results of experiments designed to test the validity of this explanation of gibberellin inhibition of rooting.

[Annals of Botany, N.S. Vol. 24, No. 96, 1960.]

METHODS

Stem cuttings from light-grown garden peas (*Pisum sativum*) and dwarf beans (*Phaseolus vulgaris*) have been used, the technique differing slightly with the two kinds of plant material.

Peas. Cuttings were taken from 2–3 weeks old plants of varieties mentioned in the text below, raised in a warm glasshouse. The plants were cut off at the second scale leaf and the first true leaf was removed. The length of each cutting was measured and it was then placed for 24 hours with the basal 3 cm. immersed in the appropriate hormone solution. The cuttings were then planted to the same depth in a mixture of equal parts of washed sand and vermiculite, moistened with Long Ashton nutrient solution and maintained at 25° C. in a water bath. After 14 days the cuttings were dug up, their length measured, and the number of roots and root initials formed counted. Twenty replicate cuttings were used for each treatment.

Dwarf beans. The technique used was essentially that of Luckwill (1956), the only differences being that in most experiments cuttings were planted in a sand: vermiculite mixture after treatment, as this was found to increase the number of roots developing and reduce variation between replicates, and that a temperature of 25° C. was used rather than the 30° C. recommended by Luckwill. The variety 'Masterpiece' was used throughout and 20 replicates were used for each treatment. Cuttings were treated by standing in appropriate solutions, so that the base of the stem was immersed up to the cotyledonary node, for 4 hours at 25° C. Certain modifications of this procedure for special purposes are described in the text.

RESULTS

Experiments with Peas

In our previous work (Brian *et al.*, 1955; Brian, 1957) we used the pea variety 'Meteor'. This produced roots very sparsely and the variation between replicates was considerable. We therefore compared a number of varieties (Table 1). Three features of the results obtained are worthy of attention. (1) Some varieties (Meteor, Alaska, Improved Pilot) rooted poorly, whereas others (Onward, Achievement) produced abundant roots; this marked varietal difference in response, noted previously by Went and Thimann (1937), was completely unrelated to differences in growth-rate, talls and dwarfs being represented in both rooting-classes. (2) Gibberellic acid (GA) reduced rooting in all varieties and at the same time increased extension of the cuttings. (3) The auxins naphthalene-acetic acid (NAA) and indolylacetic acid (IAA) increased rooting in some, but not all, varieties; similarly auxins inhibited the extension of cuttings of some varieties but not of others, and stimulation of rooting was not necessarily associated with inhibition of extension.

A factorial experiment with various levels of gibberellic acid and indolylacetic acid was therefore carried out with the readily rooting variety 'Onward'

TABLE I

Effect of Auxins and Gibberellic Acid on Rooting and Extension of Cuttings of Several Varieties of Pea, 14 days after Treatment

[Values marked * significantly different from untreated, $P < 0.05$, based on analysis of transformed data]

Variety	Treatment and concentration ($\times 10^{-4}$ M)	Mean no. roots per cutting	No. of cuttings rooted	Mean increase in length (mm.)
Meteor (dwarf)	None	0·9	6	38
	NAA (0·5)	3·1*	13	23*
	IAA (0·5)	3·7*	13	29*
	GA (0·5)	0·3*	3	90*
	GA (1·0)	0·0*	0	104*
Onward (dwarf)	None	8·4	14	36
	NAA (0·5)	13·3*	15	36
	IAA (0·5)	12·4*	14	46
	GA (0·5)	6·9	15	137*
	GA (1·0)	0·1*	13	170*
Alaska (tall)	None	0·9	5	71
	NAA (0·5)	0·9	5	51*
	IAA (0·5)	0·8	4	75
	GA (0·5)	0·2*	1	118*
	GA (1·0)	0*	0	123*
Achievement (tall)	None	14·1	15	52
	NAA (0·5)	13·0	15	44
	IAA (0·5)	18·6	15	52
	GA (0·5)	6·3*	13	91*
	GA (1·0)	2·0*	11	93*
Improved Pilot (tall)	None	4·3	13	57
	NAA (0·5)	3·2	12	46
	IAA (0·5)	4·7	13	37*
	GA (0·5)	2·2*	10	108*
	GA (1·0)	0·5*	6	117*

(Tables 2 and 3). As in earlier experiments the auxin increased rooting and gibberellic acid reduced it; analysis of variance of transformed data showed that the IAA × GA interaction was not significant. Gibberellic acid increased extension, but indolylacetic acid had no effect and the IAA × GA interaction was not significant. Thus, though the effects of gibberellic acid on rooting and on extension were opposed, and could by themselves be regarded as supporting the thesis that apical extension and basal rooting are competitive, the effect of IAA could not be similarly explained. This result differs from earlier ones (Brian, 1957) with the variety 'Meteor', where IAA did suppress elongation of cuttings. In this respect 'Onward' does appear to behave differently (see Table 1). However, the error variance with peas was found to be undesirably high and subsequent investigations were carried out with dwarf-bean cuttings which were found to be more uniform in their behaviour.

TABLE 2

Effect of Indolylacetic and Gibberellic Acids on Rooting of Onward Pea Cuttings; No. of Roots per Cutting, 14 days after Treatment

Gibberellic acid (μg./ml.)	Indolylacetic acid (μg./ml.)			Mean
	0	1	10	
0	14·7	19·0	43·2	25·6
0·01	7·7	22·4	42·8	24·3
0·1	1·8	8·4	31·5	13·9
1·0	3·2	5·6	19·2	9·3
Mean	6·9	13·9	34·2	—
	Transformed data ($\sqrt{x+0·5}$)			
0	3·90	4·41	6·61	4·97
0·01	2·86	4·78	6·58	4·74
0·1	1·52	2·99	5·66	3·39
1·0	1·92	2·46	4·44	2·94
Mean	2·55	3·66	5·82	—

Significant differences:	Individual entries	IAA means	GA means
$P = 0·05$	0·98	0·49	0·57
$P = 0·01$	1·29	0·65	0·75

TABLE 3

Effect of Indolylacetic and Gibberellic Acids on Extension (mm.) of Onward Pea Cuttings, 14 days after Treatment

Gibberellic acid (μg./ml.)	Indolylacetic acid (μg./ml.)			
	0	1	10	Mean
0	46	43	48	46
0·01	52	60	42	51
0·1	86	98	85	90
1·0	128	115	123	122
Mean	78	79	75	—

Experiments with Dwarf Beans

Auxin-gibberellin interactions. Results of a factorial experiment with indolylacetic and gibberellic acids are presented in Table 4. Analysis of variance of transformed data showed that both substances significantly ($P < 0·001$) affected both rooting and shoot extension, and that in each case the IAA × GA interaction was significant ($P < 0·01$). Gibberellic acid reduced rooting and enhanced extension, whereas indolylacetic acid had an opposite effect. The effects of gibberellic and indolylacetic acids were less than additive, gibberellic acid to a great extent blocking the rooting response to indolylacetic acid non-competitively, indolylacetic acid similarly blocking the extension response to gibberellic acid. These results could be considered to support strongly the hypothesis that extension and rooting are competitive and, indeed, it will be seen from Fig. 1 that the relation between rooting and extension of cuttings in this experiment is remarkably precise.

TABLE 4

Effect of Indolylacetic and Gibberellic Acids on Rooting and Extension of Masterpiece Bean Cuttings, 10 days after Treatment

Original data

Gibberellic acid (µg./ml.)	Mean no. of roots per cutting Indolylacetic acid (µg./ml.)			Mean increase in cutting length (mm.) Indolylacetic acid (µg./ml.)		
	0	50	100	0	50	100
0	15·4	35·0	87·4	56	26	12
1	4·4	15·1	36·3	121	50	29
10	3·0	9·6	15·0	147	68	48

Transformed data ($\sqrt{x}$ *for root no.;* log x *for cutting length*)

0	3·92	5·92	9·35	1·748	1·407	1·019
1	2·10	3·89	6·03	2·081	1·697	1·455
10	1·73	3·10	3·87	2·167	1·831	1·681

Significant differences (transformed data):

	Root no.	Cutting length
$P = 0·05$	0·91	0·085
$P = 0·01$	1·19	0·113

TABLE 5

Effect of Indolylacetic and Gibberellic Acids on Rooting and Extension of Masterpiece Bean Cuttings, 7 days after Treatment

Original data

Gibberellic acid µg./ml.	Mean no. of roots per cutting		Mean increase in cutting length (mm.)	
	No IAA	50 µg./ml. IAA	No IAA	50 µg./ml. IAA
0	41·2	88·6	32	18
0·1	20·8	52·6	31	19
1·0	18·5	37·6	41	25
10·0	13·4	24·9	68	27

Transformed data ($\sqrt{x}$ *for root no.;* log x *for cutting length*)

0	6·42	9·41	1·54	1·255
0·1	5·55	7·25	1·494	1·272
1·0	4·30	6·13	1·613	1·397
10·0	3·66	4·99	1·830	1·426

Significant differences (transformed data):

	Root no.	Cutting length
$P = 0·05$	0·84	0·107
$P = 0·01$	1·10	0·142

Table 5 gives the data for a similar experiment, including a much lower dose of gibberellic acid than in previous experiments. The results were generally similar and the data fall reasonably well on the same curve in Fig. 1. However, the lowest gibberellic acid dosage (0·1 µg/ml.) significantly reduced

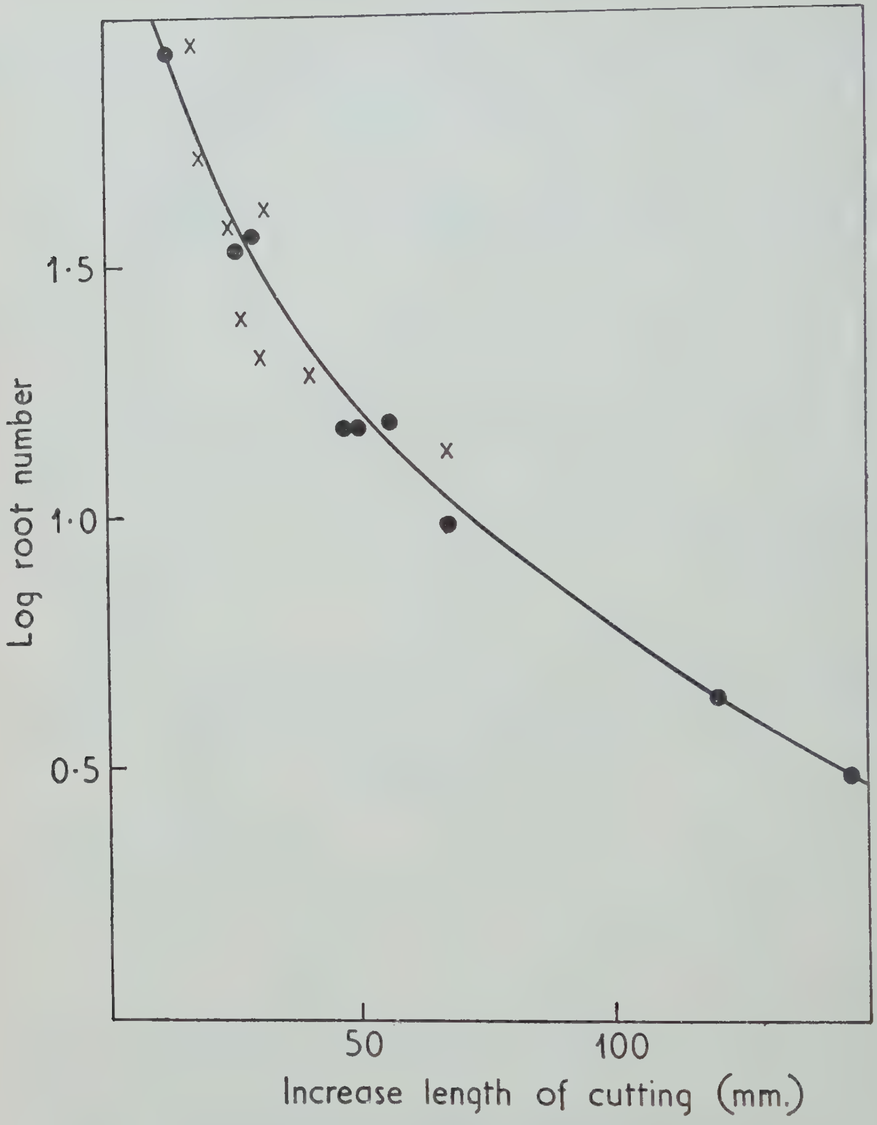

FIG. 1. Relation between root number and extension of *Phaseolus* cuttings. [● Data from Table 4; × Data from Table 5]

the number of roots formed without increasing extension of cuttings. This was confirmed in several other experiments and led us to doubt whether the negative correlation between rooting and stem extension was physiologically meaningful. Accordingly further experimental confirmation was sought.

Effect of disbudding cuttings. The standard bean cutting used in these experiments has an apical bud in the axil of the first pair of simple leaves. Nearly all the increase in cutting length recorded in Tables 4 and 5 was due to extension of new internodes below this bud and above the first leaf pair. Thus by excising the apical bud at the beginning of an experiment, and any lateral buds developing during the experiment, shoot extension can be prevented, or reduced to not more than 5 mm. Table 6 shows the results of an experiment in which gibberellin-auxin interactions were studied using intact cuttings and disbudded cuttings.

TABLE 6

Effect of Indolylacetic and Gibberellic Acids on Rooting of Intact and Disbudded Masterpiece Bean Cuttings, 7 days after Treatment

Original data (mean no. of roots per cutting)

Gibberellic acid µg./ml.	Intact cuttings			Disbudded cuttings		
	No IAA	50 µg./ml. IAA	*Mean*	No IAA	50 µg./ml. IAA	*Mean*
0	42·2	80·0	*61·2*	50·1	58·9	*54·5*
0·1	33·4	52·2	*42·8*	28·7	51·4	*40·4*
1·0	26·9	35·4	*31·2*	25·2	35·9	*30·6*
Mean	*34·2*	*55·9*	—	*34·7*	*48·7*	—

Transformed data ($\sqrt{x}$)

Gibberellic acid µg./ml.	No IAA	50 µg./ml. IAA	*Mean*	No IAA	50 µg./ml. IAA	*Mean*
0	6·50	8·95	*7·72*	7·08	7·68	*7·38*
0·1	5·78	7·23	*6·50*	5·36	7·17	*6·27*
1·0	5·19	5·95	*5·57*	5·02	5·99	*5·51*
Mean	*5·82*	*7·37*	—	*5·82*	*6·95*	—

	Individual entries	Horizontal means	Vertical means
Significant differences $P = 0·05$:	1·16	0·67	0·83
(transformed data) $P = 0·01$:	1·54	0·89	1·09

Analysis of variance of transformed data showed that auxin and gibberellic acid main effects were highly significant, that disbudding had no effect and that no interactions reached significance. Thus gibberellic acid reduced rooting of disbudded cuttings to the same extent as in intact cuttings, despite the fact that disbudding prevented any appreciable extension of cuttings. In other words, the two characteristic effects of gibberellin have been dissociated and reduction of rooting has been shown to be independent of increased extension.

Apical application of gibberellic acid. In a further experiment (Table 7), the auxin treatment of cuttings was as usual basal, but the gibberellic acid was applied to the apical bud by the alcohol microdrop method (Brian and Hemming, 1955). Under such circumstances 0·1–10 µg. gibberellic acid increased shoot extension considerably but did not affect rooting in any way. Thus the two effects of gibberellic acid have again been dissociated, in this case showing that increased extension of the cuttings does not necessarily reduce rooting. The failure of gibberellic acid to reduce rooting when applied apically is probably a consequence of slow or inadequate downward translocation. Larger doses applied apically do reduce rooting (Table 8); presumably

TABLE 7

Effect of Indolylacetic Acid (Basal) and Gibberellic Acid (Apical) on Rooting and Extension of Masterpiece Bean Cuttings, 10 days after Treatment

Original data

Gibberellic acid (µg./cutting)	Mean no. of roots per cutting Indolylacetic acid (µg./ml.)			Mean increase in cutting length (mm.) Indolylacetic acid (µg./ml.)		
	0	50	100	0	50	100
0	46·9	100·2	129·5	31	22	12
0·1	60·3	111·2	137·9	69	54	42
1·0	45·0	96·3	132·4	135	88	69
10·0	47·1	98·0	122·5	162	89	63

Transformed data ($\sqrt{x}$ *for root no.,* log x *for cutting length)*

Gibberellic acid (µg./cutting)	0	50	100	0	50	100
0	6·85	10·01	11·38	1·483	1·341	1·076
0·1	7·09	10·54	11·74	1·836	1·732	1·624
1·0	6·73	9·81	11·51	2·131	1·944	1·840
10·0	6·86	9·91	11·07	2·209	1·951	1·801

Significant differences (transformed data):

	Root no.	Cutting length
P = 0·05	0·77	0·110
P = 0·01	1·02	0·146

TABLE 8

Effect of Time of Application of Gibberellic Acid on Rooting of Masterpiece Bean Cuttings

(Mean no. of roots per plant, 10 days after planting cuttings)

Original data

Gibberellic acid (µg./plant)	Time of treatment*				
	−48	−24	0	+24	+48
0	22·0	19·7	24·8	23·7	14·4
100	7·2	11·4	11·9	13·1	18·4
500	6·5	8·4	8·3	11·1	20·9

Transformed data ($\sqrt{x}$*)*

Gibberellic acid (µg./plant)	−48	−24	0	+24	+48
0	4·70	4·44	4·98	4·87	4·57
100	2·76	3·28	3·44	3·62	4·29
500	2·55	2·89	2·89	3·23	3·80

Significant differences (transformed data)

P = 0·05:0·59
P = 0·01:0·79

* Hours before or after cutting taken and planted in tap water.

under such circumstances a sufficient concentration accumulates at the base to have an inhibitory effect.

Time of application of gibberellic acid. Plants were treated apically with gibberellic acid (or with alcohol alone) 48 hours and 24 hours before the shoots were cut off, and also at the time of cutting and 24 and 48 hours afterwards. These cuttings were rooted in tap water. No roots had formed but some primordia were already visible at the last time of treatment. The highly significant ($P < 0{\cdot}01$) interaction between gibberellic-acid treatment and time of application in this experiment (Table 8) was a reflection of the fact that while the alcohol controls gave similar results whatever the time of treatment, the gibberellic-acid treatments were most effective when application was made early; a 100μg. dose which was highly inhibitory when applied to the plant 48 hours before the cutting was taken did not significantly reduce rooting when applied 48 hours after the cutting was taken. Similar results have been obtained in experiments in which gibberellic acid was applied basally.

Effect of gibberellic acid in the presence of toxic doses of auxin. If, instead of a 4-hour pre-treatment with indolylacetic acid, the cuttings are left permanently in the solutions, rooting is reduced and much disorganized cell-proliferation takes place in the cortex leading to splitting and distortion of the base of the cutting. Table 9 gives the results of an experiment in which cuttings were kept continuously in IAA and gibberellic acid, the solutions being replaced by fresh ones every 3 days. Under such circumstances both IAA and gibberellic acid reduced rooting, though their effects were less than additive. Cortical proliferation induced by IAA was not reduced by gibberellic acid. IAA severely restricted stem extension.

TABLE 9

Effect of Continuous Treatment of Masterpiece Bean Cuttings with Indolylacetic and Gibberellic Acids on Rooting and Extension, 10 days after Beginning of Treatment

Original data

Gibberellic acid (μg./ml.)	Mean no. of roots for cutting Indolylacetic acid (μg./ml.)			Mean increase in cutting length (mm.) Indolylacetic acid (μg./ml.)		
	0	25	100	0	25	100
0	15·0	29·6	5·1	51	17	10
1·0	3·4	14·0	2·1	152	19	22
10·0	3·1	5·9	1·6	151	42	26

Transformed data ($\sqrt{x+0{\cdot}5}$ *for root no.*, log x *for cutting length*)

Gibberellic acid (μg./ml.)	Root no. 0	Root no. 25	Root no. 100	Cutting length 0	Cutting length 25	Cutting length 100
0	3·94	5·49	2·37	1·703	1·223	1·000
1·0	1·97	3·81	1·61	2·180	1·281	1·332
10·0	1·90	2·10	1·45	2·178	1·618	1·412

Significant differences (transformed data):

	Root no.	Cutting length
$P = 0{\cdot}05$	1·28	0·150
$P = 0{\cdot}01$	1·70	0·199

Comparison of gibberellic acid with gibberellin A_1. It was conceivable that gibberellic acid inhibited root initiation because of a structural difference from the native gibberellins of the bean. Gibberellin A_1 has been identified in *Phaseolus* seed (Macmillan and Suter, 1958; West and Murashige, 1958). We have therefore compared these two substances, but both inhibit root initiation to a similar extent (Table 10).

TABLE 10

Effect of Gibberellin A_1 and Gibberellic Acid on Rooting of Masterpiece Bean Cuttings, 10 days after Treatment

(*Mean number of roots per cutting*)

	Concentration (µg./ml.)			
	0	0·1	1·0	10·0
Gibberellin A_1	16·9	12·2	7·7	3·2
Gibberellic acid		10·7	7·3	3·6

Attempted reversal of gibberellic acid inhibition. A number of substances other than auxins have been tested in combination with gibberellic acid. These included some which have been reported to enhance root initiation, viz. adenine, adenosine (Galston and Hand, 1949), guanine, biotin (Went and Thimann, 1937), and, in addition, kinetin (6-furfurylaminopurine) and several sulphhydryl compounds. None of these affected rooting under our conditions, nor did they in any way reduce the inhibition caused by gibberellic acid.

DISCUSSION

The results presented above show that gibberellic acid has two effects on pea or bean cuttings: (*a*) extension of the cuttings is stimulated; (*b*) formation of adventitious roots is inhibited. It has also been shown that, though under many circumstances these two effects occur together, they can be experimentally dissociated. This dissociation has been observed under the following circumstances: (*a*) small doses of gibberellic acid applied basally partially inhibit rooting without increasing stem extension; (*b*) if bean cuttings are disbudded, thus preventing any marked stem extension, this in no way affects the rooting inhibition by gibberellic acid; (*c*) small doses of gibberellic acid applied apically greatly increase extension of the cuttings without affecting root initiation. Thus cutting extension is not dependent on the presence of roots; in our experiments with beans greatest extension has been observed in cuttings treated with heavy doses of gibberellic acid where no roots were formed at all. In so far as it induces extension of cuttings, gibberellic acid may be considered to have properties similar to the caulocaline of Went (1938). Further, inhibition of rooting by gibberellic acid is now shown to be entirely independent of cutting extension, and the nutrient diversion hypothesis previously put forward (Brian, 1957) must be abandoned.

At present no alternative explanation of rooting inhibition by gibberellic acid can be offered. The faint possibility that gibberellic acid might in this system act as an antagonist to a natural gibberellin present in the cuttings may be discounted since it has been shown that gibberellin A_1, known to be present in *Phaseolus* (Macmillan and Suter, 1958), also inhibits rooting. Nevertheless, the nature of this gibberellin effect is a little clearer than it was. The difference in response to apically and basally applied gibberellic acid strongly suggests that the gibberellic acid must be present in the tissues of the stem base if rooting is to be inhibited. In other words, the inhibition is a direct local effect. Gibberellic acid is less inhibitory to rooting if application is delayed until after the initial divisions involved in the formation of primordia have taken place; thus its effect is primarily to inhibit this first stage in root formation. Certainly, once primordia have been formed gibberellic acid is powerless to prevent further development. This view is supported by direct observation of sections of cuttings and by the earlier observation (Brian, Hemming, and Radley, 1955) that gibberellic acid does not inhibit root extension. The inhibition of rooting of *Salix gmelinii* and *S. purpurea* reported by Gundersen (1958) calls for comment here, because in these plants root primordia were presumably already present in the stem tissues at the time of treatment. However, in his experiments, though the cuttings were repeatedly treated with gibberellic acid, reduction in rooting was far less marked than in our experiments with beans and peas where a single initial dose was used. Thus it seems reasonable to conclude that a local concentration of gibberellin in the base of the stem prevents meristemization of adult stem tissues.

In this respect gibberellic acid has an opposite effect to that of auxins, but our results do not suggest that there is a competitive gibberellin: auxin relationship. Rather gibberellins block the response to auxin. Though synergistic relations between auxins and gibberellins are more common (Brian, 1959), antagonistic relations, other than the one we are dealing with, are known, e.g. in stem extension of *Impatiens* (Weijer, 1959) and less strikingly in stem extension of the pea and bean cuttings used in our rooting experiments, though in these cases it is the auxin which is inhibitory. Again, though gibberellic acid is known to induce cell-division in many circumstances (Brian, 1959), it is also known to inhibit cell-division on occasion, as in the inhibition of root-nodule formation on leguminous plants (Galston, 1959; Thurber, Douglas, and Galston, 1958) and in inhibition of proliferation of some tissue cultures (Nickell and Tulecke, 1959). Thus the inhibition of adventitious rooting is not an entirely isolated phenomenon. It is, however, rather surprising that gibberellic acid in no way inhibits the disorganized cortical cell-proliferation induced by massive doses of indolylacetic acid.

One further observation made in the course of these experiments is worthy of further discussion. It is now well established that gibberellic acid will induce most genetic dwarfs to assume the phenotypic habit of wild-type tall plants. This was first observed in peas (Brian and Hemming, 1955) and led to the suggestion (Radley, 1956; Brian, 1957) that dwarf peas might differ from talls in having an impaired mechanism of gibberellin synthesis. If that were so one might expect cuttings from tall peas, with a high endogenous gibberellin level, to root more poorly than cuttings from dwarfs. We have shown that this is not so; though marked varietal differences in ease of rooting do exist in peas, this variation is not correlated with the rate of stem extension, talls and dwarfs appearing in any groupings based on ease of rooting. Radley (1958) has shown that in fact roughly similar amounts of gibberellin can be extracted from the basal portions of stems of Meteor (dwarf) and Improved Pilot (tall) peas. The rooting response of these two varieties (Table 1) is also similar, both rooting rather sparsely.

SUMMARY

1. Peas showed marked varietal differences in the ease of rooting of stem cuttings. Gibberellic acid increased extension and inhibited rootings of all varieties tested.

2. Similar results were obtained with dwarf beans (*Phaseolus*), and in many factorial experiments in which combinations of gibberellic acid and indolylacetic acid were studied, the amount of rooting was inversely correlated with stem extension, apparently supporting the hypothesis that inhibition of rooting by gibberellin could be due to diversion of essential nutrients to the extending apical regions of the cutting.

3. This hypothesis had nevertheless to be abandoned because it was found experimentally possible to dissociate effects on stem extension and on root formation in three ways. (*a*) Small doses of gibberellic acid applied basally reduced rooting without increasing extension. (*b*) Small doses of gibberellic acid applied apically markedly increased extension of cuttings without reducing rooting. (*c*) If cuttings were disbudded, thus preventing extension, gibberellic acid nevertheless reduced root initiation as much as it did on normal cuttings.

4. The effectiveness of a dose of gibberellic acid was greatest if applied shortly before the cutting was removed from the plant, and decreased in effectiveness sharply if applied two days after the cutting was taken. It is believed that gibberellic acid prevents rooting by a direct local inhibition of those cell divisions in the natural stem tissues which precede the formation of organized root primordia.

5. The antagonism between gibberellic acid and auxins is non-competitive. Gibberellin A_1, known to occur naturally in *Phaseolus*, is as active as gibberellic acid in inhibition of rooting of *Phaseolus* cuttings.

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Studies on the Physiology of Nodule Formation

VII. A Reappraisal of the Effect of Preplanting

BY

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With two Figures in the Text

ABSTRACT

Nutman (*Ann. Bot.* **21**, 321, 1957) found that preplanting agar slopes with *Trifolium pratense* L. or *Medicago sativa* L. advanced the time when second lots of plants of these species grown on the same slopes initiated nodules, and depressed the total number of nodules formed. He attributed these effects to root secretions which at low concentration hasten initial nodulation but at higher concentrations inhibit nodule formation. Further work has now shown that initial nodulation is stimulated because the preplant removes traces of nitrate from the medium. The amount of nitrate in the tap water used to prepare the medium (6·5 p.p.m. N) also increases the number of nodules formed on the control plants, and this effect explains to a considerable extent the depression of nodule numbers by preplanting.

Initial nodulation was delayed by small amounts of nitrate and nitrite but not by other forms of combined nitrogen (ammonium, asparagine, and urea). All forms of combined nitrogen tested increased the number of nodules formed over a period of 8 weeks when supplied at an initial concentration of 20 p.p.m. N.

INTRODUCTION

ALTHOUGH the various stages of nodule initiation and nodule formation have been determined by cytological studies, the physiological and biochemical factors controlling these stages are poorly understood. From the results of experiments in which plants of the same, or different, species were grown in association, several workers have suggested that root secretions from adjacent plants may stimulate nodule formation on young seedlings; this stimulation applies to both the time interval between germination and the appearance of the first nodule on a root (time of initial nodulation) and to the number of nodules formed during the early stages of seedling development (Thornton, 1929; Ludwig and Allison, 1935; Nutman, 1953). Further, from studies of the physiology of nodule formation, Nutman (1945, 1948, 1952*a*) suggested that high concentrations of root secretions may decrease the number of nodules formed on older seedlings.

To eliminate the competitive effects obtained when plants are grown in association, and to study the effect of root secretions on nodule initiation and

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development, Nutman (1953, 1957) developed a preplanting technique which separated the donating and reacting components. *Medicago sativa* L. (lucerne) and *Trifolium pratense* L. (red clover) were grown on agar slopes for varying periods, and after removing these 'donor' plants, inoculated seeds were germinated and grown on the same slopes. The results of these experiments suggested that the donor plants secreted substances that advanced initial nodulation by 4–7 days. This stimulation of nodule initiation by preplanting was followed by a decrease in the total number of nodules formed by the test plants. Nutman concluded from these experiments that the roots of the preplants secreted an active agent (or agents) which accumulated in the medium and at low concentration stimulated nodule inception and at higher concentrations inhibited nodule formation; the production of, and response to, the agent depended on the species of donor and test plant. Support for this inhibition hypothesis seemed to come from the fact that adding adsorbents such as charcoal and bentonite to the medium increased the number of nodules formed (Nutman, 1952*b*). Turner (1955) confirmed this effect with charcoal but also found that initial nodule formation on red clover was stimulated by charcoal. As this effect was assumed to come from charcoal adsorbing the inhibitory root secretions, this observation appeared to be at variance with Nutman's conclusion that root secretions stimulate initial nodule formation.

The work now to be described was done to examine the effects of root secretions on nodule formation in an attempt to find the site of their physiological activity.

MATERIALS AND METHODS

All plants were grown under sterile conditions on mineral nutrient agar slopes in uniform $6 \times \frac{3}{4}$ in. test-tubes. The technique was basically the same as that used by Thornton (1930) and Nutman (1948) but the methods of media preparation and seed handling were modified.

The principal seedling nutrient solution used was based on that formulated by Thornton (1930). As most of the calcium phosphate and ferric phosphate in the medium is precipitated within the tubes and interferes with plant observation, these salts were brought to $\frac{1}{4}$–$\frac{1}{5}$ of that normally used by preparing a stock solution as follows: 200 g. of $Ca_3(PO_4)_2$ and 50 g. $FePO_4$ were thoroughly shaken in 2 l. distilled water in a 3 l. conical flask, and allowed to stand 10 minutes before decanting the cloudy supernatant fluid, which was made up to 2 l. and used for all media at the rate of 20 ml./l. (Nutman usually decreased the amount of precipitate by decanting the medium before dispensing.) In one experiment the agar slopes were prepared with Jensen's nutrient salt solution (Jensen, 1942).

Unless otherwise stated, Difco Bacto-agar and distilled water were used for all media, to which was added 1·0 ml./l. of the following trace-element solution: H_3BO_3 (2·86 g./l.), $MnSO_4.H_2O$ (2·03 g./l.), $ZnSO_4.7H_2O$ (0·22 g./l.), $CuSO_4.5H_2O$ (0·08 g./l.), and Na_2MoO_4 (0·11 g./l.). The pH was adjusted to 6·6 before the medium was dispensed into test-tubes (8 ml./tube). The tubes were plugged with cotton wool to prevent contamination, but the plugs were loose enough to allow gas diffusion. The tubed medium was autoclaved at 15 lb. pressure for 20 minutes and sloped with the top of the agar 3–3½ in. above the bottom of the tube. The final pH of the medium was 6·7.

The plant species used were *Trifolium pratense* L., selected families and commercial seed of Montgomeryshire late-flowering red clover; *T. repens* L., strain S100 from the Plant Breeding Station, Aberystwyth; *Medicago sativa* L., Provence variety. The bacterial strains used were *Rhizobium trifolii* Dang., strain Clover F, and *Rh. meliloti* Dang., strains AH_1 (identical with AH_2), SU 277/1, CC 148, and SU 47, all of which fix nitrogen in symbiosis with their appropriate hosts.

After removing all large, small, abnormal and damaged seeds, each sample of seeds was placed in a desiccator overnight, and surface sterilized the following morning with conc. H_2SO_4 (20 minutes for lucerne and white clover, 30 minutes for red clover). After thorough washing, the seeds were placed on sterile distilled-water agar (1 per cent.) in Petri dishes which were inverted during seed germination. After 24–30 hours at 25° C., germinated seedlings with radicles ¼–½ in. long were transferred to seedling nutrient agar slopes so that the cotyledons were 2¾ in. above the bottom of the tube, i.e. ¼ in. above the top of the wooden racks in which the tubes were then placed. After sowing, the tubes remained at room temperature overnight. The next morning the contents of a 3-day culture of the appropriate strain of rhizobia were added to 750 ml. of ¼-strength seedling nutrient solution, and 2 ml. of this solution was added to each tube. By this method each tube received approximately 10^7 viable organisms. The water level was marked, and the level maintained by adding sterile nutrient solution periodically. After watering, the tubes were allocated at random to statistical blocks, and placed in the glasshouse. The next day the inoculated suspension in each tube was rinsed over the roots of the young seedlings.

The age of the seedlings was calculated from 9 a.m. on the day they were sown; this was day 0. Observations for initial nodules started on day 5, and continued daily until the plants were nodulated. At each observation the position of any structure resembling a nodule was recorded, and the observation verified or not within the next 3 days. On day 5 all poorly developed seedlings, and all seedlings with roots growing into the agar slope at a point more than 1 in. above the water level, were discarded, as these plants frequently nodulated abnormally late.

The glasshouse was heated in winter and during the summer the temperature in the test-tubes was maintained below 30° C. by judicious shading with slatted roof-blinds, by ventilation, and by watering the floor. For one experiment, the plants were grown in a controlled environment cabinet with the temperature of 22° C. when in the light and 14° C. when in the dark; light was provided by fluorescent tubes (Philips's 4 ft. 40 W. Warm White) between, and 2½ in. from, the growth tubes (light intensity approx. 1,000 f.c.).

In the preplanting experiments, the entire donor plants were removed aseptically, and those tubes with pieces of root remaining in the agar were discarded. During the preplanting phase the unsown control slopes were included in the experimental layout in the glasshouse.

In experiments where nitrate was added to the medium, 0·5 ml. of a sterile solution of KNO_3 was added to the tubes after sowing. For the charcoal treatments, Sutcliffe–Speakman wood charcoal was washed successively in conc. H_2SO_4, 40 per cent. NaOH and acetone, then rinsed twelve times in distilled water before drying at 60° C. The charcoal was weighed and sterilized on small aluminium-foil shovels for 10 hours at 130–150° C., and carefully deposited at the bottom of the agar slopes after the plants were sown.

Total nitrogen determinations: The dried plant material was incinerated in 25 ml. Kjeldahl flasks with 2 ml. Analar H_2SO_4 and 0·2 g. of an 8 : 1 mixture of K_2SO_4 and $CuSO_4$. The material was digested for 2 hours after clearing. The ammonia in the digest was distilled from a Markham still into 2 per cent. boric acid (w/v), and this was back-titrated with N/100 HCl.

RESULTS

The time of initial nodulation

A number of preplanting experiments failed to repeat the degree of stimulation of initial nodulation found by Nutman (1957) with red clover and lucerne (Table 1). These experiments also showed that the time of initial nodulation was not hastened by adding 0·1 g. charcoal to the agar medium.

TABLE 1

The Effect of Preplanting on the Time of Initial Nodulation, in Days, of Lucerne and Red Clover

Species	Line	Control	Preplanted	0·1 g. charcoal	Preplanting +charcoal
Red clover	Early nod.	12·4	12·2	10·0	9·9
	Late nod.	13·4	14·4	13·9	11·3
	Sparse nod.	10·6	11·2	11·6	10·0
	Abundant nod.	9·8	9·0	9·3	8·1
Red clover	Commercial	8·0	7·9	—	—
Lucerne	Provence	13·0	11·8	14·0	12·1
Lucerne	Provence	14·0	16·8	—	—
	Provence*	8·4	9·0	—	—
Lucerne	Provence†	9·6	—	10·7	—

* Inoculated SU277/1; other lucerne treatments inoculated AH_1.
† Mean 3 treatments, inoculated CC148, SU277/1 and SU47.
Data within this table taken from 5 experiments. Preplanting periods were 20, 15, 20 and 20 days respectively. All means based on 8–12 replicates.

The reasons for the differences between these results and those previously obtained by Nutman were sought in experiments designed to examine the effects of differences between the experimental techniques used on the two occasions. Differences in the methods of seed treatment and handling did not affect the time of initial nodulation; nor were there any differences when we both examined the plants and recorded nodules independently. However, preplanting on media prepared according to Nutman's original formulation promoted earlier nodule initiation in red clover and lucerne. Nodules were initiated earlier on plants growing on the medium described above than on Nutman's medium.

Experiment 1

The principal differences between the two media were the sources of agar and water: Nutman's medium contained Hopkins and Williams's shredded agar and tap water, whereas the new formulation contained Difco Bacto-agar and distilled water. In a 3×2^4 factorial experiment designed to compare the effect of these different components of the media, the experimental treatments were: (i) Bacto-agar *v.* Shredded agar. (ii) Distilled water *v.* tap water. (The inorganic nutrient solution was the same for the four media types.) (iii) No preplanting *v.* 18 days preplanting. (iv) Inoculation at sowing *v.* 18 days preinoculation. (v) Host species: Red clover, white clover (inoculated Clover F), and lucerne (inoculated AH_1).

Slopes preplanted with one species were test-planted with the same species. The experiment began in mid-April 1958, and continued until early July 1958. For the test planting, ten replicates of each treatment were sown.

Analysis of the data showed a high order of interaction between the five major treatments, and two of the third-order interactions were significant at the 5 per cent. level. However, the main differences concerned preplanting and water-type. Table 2 shows the means of all treatments; those in which the agar source and inoculation time are combined are in heavy type. Plants of each species grown on the 'tap-water control' medium nodulated significantly later than those in the other treatments. Preplanting the 'distilled water' medium never promoted significantly earlier nodulation; preplanting the 'tap-water' medium stimulated initial nodulation to a degree similar to that reported by Nutman (1957). The effect of preinoculation and agar-type are discussed later.

These results were interpreted as indicating that the tap water contained something that delayed initial nodulation and was removed by preplanting. Total-nitrogen determinations on uninoculated preplants from each of the main treatments indicated that the plants grown on the tap-water medium assimilated 60 μg. N during 18 days' growth, whereas those grown on the distilled-water medium assimilated no combined nitrogen. Further analysis of the tap water showed that it contained 6·5 p.p.m. nitrate-nitrogen, i.e. 65 μg. N per 10 ml. medium.

The inhibitory effect of combined nitrogen on nodule formation is well known (Fred, Baldwin and McCoy, 1932; Wilson, 1940), but there is no information in the literature to suggest that the small amount of nitrate present in the tap water could delay initial nodulation.

TABLE 2

The Effect of Media-Type and Pretreatment on the Time of Initial Nodulation, in Days, of Lucerne, Red Clover and White Clover

			Control			Preplanted		
Species	Water	Agar	Control		Preinoc.	Control		Preinoc.
Red clover	Dist.	Bacto	7·9	**7·4**	7·2	7·9	**7·7**	8·4
		Shredded	7·5		7·1	7·6		7·1
	Tap	Bacto	10·7	**9·7**	10·1	8·5	**8·0**	8·4
		Shredded	8·9		9·1	7·8		7·2
White clover	Dist.	Bacto	7·9	**7·4**	7·4	7·2	**7·0**	7·1
		Shredded	7·2		7·4	7·3		6·3
	Tap	Bacto	12·0	**10·4**	12·1	9·1	**8·1**	7·8
		Shredded	8·7		7·4	9·0		8·0
Lucerne	Dist.	Bacto	9·9	**10·3**	10·0	11·3	**10·2**	10·2
		Shredded	11·7		9·7	10·6		8·9
	Tap	Bacto	16·7	**17·0**	17·2	12·1	**10·6**	9·4
		Shredded	20·2		14·0	10·3		10·6

Values in heavy type are the species × water × preplanting treatment means. Difference for significance: 0·9 d. ($P = 0{\cdot}05$.) Difference for significance between means in small type: 1·9 d. ($P = 0{\cdot}05$.)

Experiment 2

To examine the effect of small amounts of nitrate, lucerne plants were grown on Thornton's modified Bacto-agar distilled-water medium to which four levels of KNO_3 were added—2, 5, 10 and 16 p.p.m. NO_3–N in 10 ml. medium. There were also two preplanting treatments (9 and 17 days), the test plants were inoculated with either SU 47 or AH_1. The preplants were not inoculated, and the experiment was done in the glasshouse during June–August 1958. After the preplanting periods, total N was determined for all preplants. Preplanting for 17 days removed all the added nitrate from the four N-treatments, but preplanting for 9 days left some nitrate in the 5, 10 and 16 p.p.m. treatments (2, 4 and 10 p.p.m. N respectively).

Although the bacterial strain effects were highly significant ($P < 0{\cdot}001$), they did not interact with the nitrate or preplanting treatments. The means of the preplanting × nitrate treatment in Fig. 1 show that the delay to initial nodulation without preplanting increased significantly with increasing concentration of nitrate above 2 p.p.m. With the '9 days preplanting' treatments, initial nodulation was delayed in tubes containing more than 2 p.p.m. NO_3–N at the time of test-planting. The delay in initial nodulation closely corresponds to the expected delay based on the level of the NO_3–N in the tubes after preplanting. The means of the five '17 days preplanting' treatments do not differ significantly, but there is an indication that the plants in the 'pre-planted-nitrate' treatments nodulated earlier than those in the 'unpreplanted, no-nitrate' control.

In conjunction with this experiment, lucerne plants were grown on Thornton's modified medium prepared with tap water. The mean time of initial nodulation was 17·2 days, or 5 days later than in the distilled-water medium. Adding 0·1 g. charcoal to the tap-water medium hastened initial nodulation by 4·6 days.

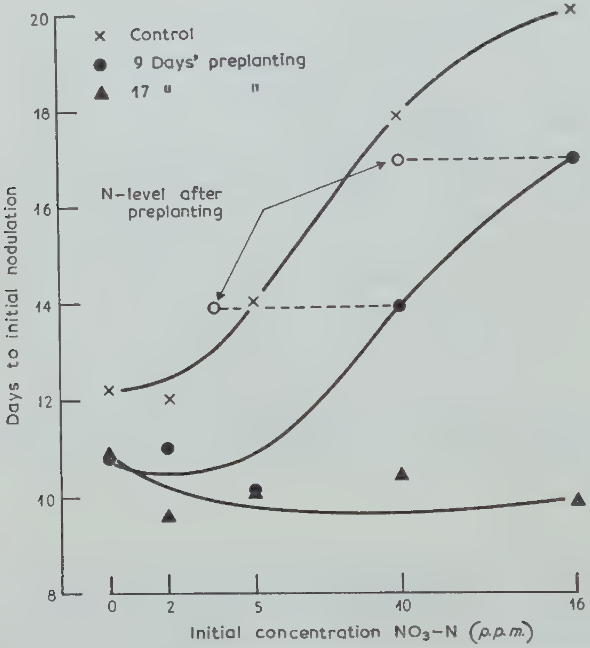

FIG. 1. The effect of combined N and preplanting on the time of initial nodulation of lucerne.

Experiment 3

An experiment was done to see whether different forms of combined N delayed initial nodulation. Hopkins and Fred (1933) supplied red clover plants with 200, 500 and 1,000 μg. N per plant (concentrations of 8, 20 and 40 p.p.m. N) as KNO_3, (NH_4), SO_4, asparagine and urea. They reported that all these forms of nitrogen delayed nodule formation, but nodule initiation was not directly recorded.

In Expt. 3, each of the four forms of combined nitrogen used by Hopkins and Fred, plus NH_4NO_3, $NaNO_3$, and $NaNO_2$, were supplied to 1-day-old inoculated white clover seedlings at the rate of 200 μg. N/tube (20 p.p.m.). The plants were grown in the controlled environment cabinet. The three nitrate salts and the sodium nitrite significantly delayed initial nodulation, but asparagine, urea and ammonium sulphate had no effect (Table 3). Total-N determinations made on four plants from each treatment on day 14 showed that nitrate, nitrite and asparagine were assimilated in approximately the same amounts, but 30 per cent. more nitrogen as urea and ammonium were assimilated in the same period (Table 3).

TABLE 3

The Effect of Combined Nitrogen on the Time of Initial Nodulation, Nodule Number (54 days) and Nitrogen Uptake (14 days) of White Clover. 200 g. N/tube

N source	Time of initial nodulation	N uptake (μg N/plant)	Nodule number
Control	7·9±0·22	0	6·7±0·86
KNO_3	11·6±0·85	85	12·6±1·58
$NaNO_3$	9·8±0·50	115	11·3±1·65
$NaNO_2$	11·0±1·01	100	12·2±3·21
NH_4NO_3	11·7±0·35	—	9·7±1·31
$(NH_4)_2SO_4$	8·3±0·27	133	9·9±0·95
Urea	7·9±0·35	135	11·3±1·98
Asparagine	7·9±0·32	90	—

Control means based on 30 observations; other treatments based on 15 observations.

The hydrogen-ion concentration of the agar slopes did not change during this 14-day period.

Nodule numbers

In the preliminary preplanting experiments nodules were counted 60–80 days after the test plants germinated. Table 4 shows that preplanting decreases the number of nodules by 20–50 per cent. Adding 0·1 g. charcoal to unpreplanted medium did not affect nodule numbers consistently, although charcoal can partly alleviate the inhibitory effects of preplanting. When added to the tap-water medium, charcoal did not affect the number of nodules developed on lucerne test plants.

TABLE 4

The Effect of Preplanting and Charcoal Treatment on Nodule Numbers

Species	Line	Control	Preplanted	0·1 g. charcoal	Preplanting +charcoal
Red clover	Early nod.	22·7	17·1	23·9	24·0
	Late nod.	13·0	9·5	18·5	18·8
	Sparse nod.	17·5	14·3	22·0	18·1
	Abundant nod.	54·1	32·0	43·5	43·0
Red clover	Commercial	12·7	9·5	—	—
Red clover	Commercial	15·5	9·2	13·7	30·5
	Commercial*	—	7·7	—	9·8
Lucerne	Provence	12·8	8·3	11·8	10·0
Lucerne	Provence	12·5	9·1	—	—
Lucerne	Provence	10·5	—	14·2	—
Lucerne	Provence†	15·1	—	13·3	—

* Preplanted with *Trifolium subterraneum* L. var. Mt. Barker.

† Plants grown on medium prepared with tap water.

Data within this table taken from 7 experiments. All means based on 8–12 replicates.

During the experiment designed to examine the effect of agar-type and water-type on initial nodulation (Expt. 1), nodules were counted at 4- or 5-day intervals until accurate counts *in situ* became impossible. A final count was made when the plants were removed from the tubes 60 days after germination. Fig. 2 shows the progress of nodulation on the plants grown on distilled-water and tap-water Bacto-agar control media. Values for the preinoculated and normally inoculated treatments are combined, as they do not differ significantly. The curves show both the extent to which nodule initiation was delayed by the nitrate in the tap water and the increase in the number of nodules actually formed. The preplanted tap-water and the preplanted distilled-water treatments gave results almost identical with those for the distilled-water controls, i.e. within 1·0 nodules per plant.

TABLE 5

The Effect of Nitrate on Nodule Number. Lucerne Plants harvested after 70 Days' Growth

Initial conc. of NO_3–N p.p.m.	Bacterial Strain	
	SU47	AH_1
0	8·0	10·0
2·0	9·2	12·4
5·0	13·9	16·3
10·0	22·1	20·0
16·0	21·6	18·3

Means based on 11 observations.

The nodules formed on the plants in the control treatments in Expt. 2 were counted 70 days after germination. Table 5 shows that small amounts of added nitrate considerably increased the total number of nodules formed on lucerne by both bacterial strains. The effect of nitrate on nodule number was confirmed in the experiment in which different sources of combined nitrogen were supplied to the plants (Table 3). Each nitrogenous compound increased the mean number of nodules significantly above the control.

DISCUSSION

The results of the first two main experiments described show clearly that the hastening of nodule initiation obtained in Nutman's preplanting experiments (Nutman, 1953, 1957) was caused not by the preplants producing a stimulant but by their removing nitrate ions from the tap water used to prepare the agar culture medium. Concentrations of nitrate–N as low as 5 p.p.m. delayed nodule initiation in lucerne, and as little as 2 p.p.m. nitrate–N delayed nodulation in both red and white clovers (Gibson, 1959). Charcoal added to the tap-water medium advanced nodulation, as found by Turner (1955) with red clover test plants. The failure of charcoal to affect the initial nodulation of lucerne or red clover grown on control medium suggests that Turner's and Nutman's (1952*b*) results may be explained by the charcoal adsorbing the inhibitory nitrate.

The inhibition of nodule formation by combined nitrogen was first observed

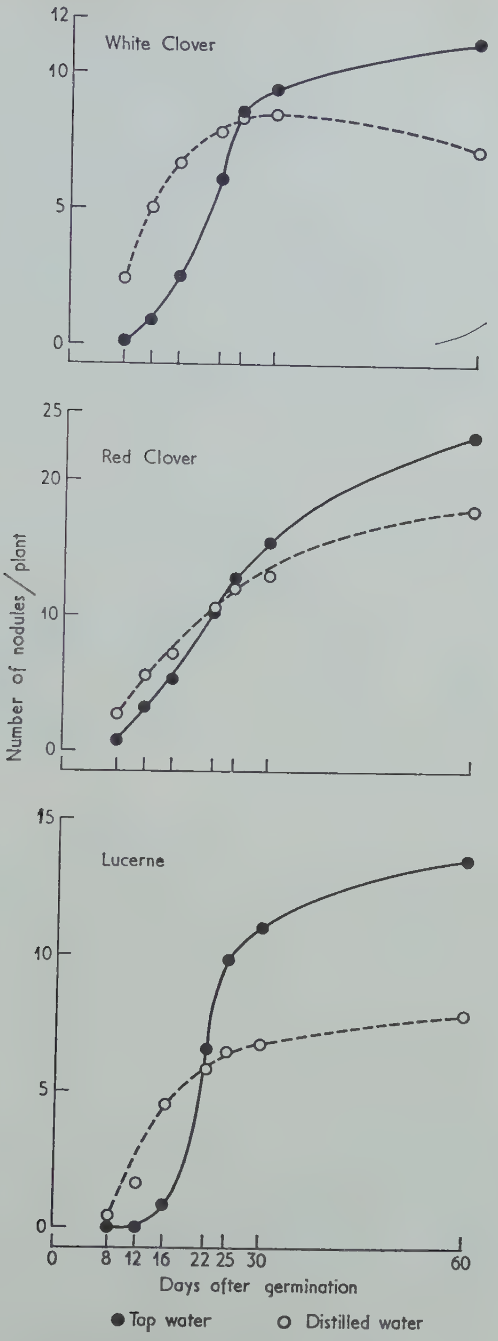

FIG. 2. The rates of nodule formation on white clover, red clover, and lucerne grown on seedling nutrient medium prepared with tap water or distilled water.

by Rautenberg and Kuhn twenty years before Hellriegel and Wilfarth demonstrated, in 1886, the relationship between nodules and nitrogen fixation (Fred, Baldwin and McCoy, 1932). Considerable research to find how combined nitrogen, mainly as nitrate, affects the nodulation of legumes, has been done, and numerous theories have been proposed to explain the effect (see Allison and Ludwig, 1934). Allison and Ludwig (1934) and P. W. Wilson (1935, 1940) consider that the amount of carbohydrate available for nodule formation is decreased because it is used during the assimilation of the combined nitrogen. However, nitrogen as ammonium sulphate, asparagine, or urea did not affect initial nodulation even though they were assimilated in approximately the same amounts as nitrate-nitrogen, which suggests that the 'available carbohydrate' or 'C: N' hypothesis is an inadequate explanation. Other experiments designed to test this hypothesis (Gibson, 1959) support the conclusion that at high dilutions of available combined nitrogen, only nitrate and nitrite ions inhibit nodule initiation.

Okhawara (1927) and Thornton (1936) suggested that small amounts of combined nitrogen, as nitrate salts, may stimulate nodule formation, but their observed increases in nodule number were small. The results presented in Table 5 show that 16 p.p.m. NO_3–N (160 μg. N per plant) delayed initial nodulation (Fig. 1) but greatly increased the number of nodules produced after 60 days. This observation explains the increased number of nodules on the 'tap-water control' plants in Expt. 1 (Fig. 2). The similarity between these results and those of Nutman (1959, Fig. 2) suggests that Nutman's preplanting effects with regard to nodule number can also be attributed to small amounts of nitrate in the medium.

Comparison of the mean times of initial nodulation with nodule number means in the present experiments shows a high degree of correlation between the two variables, and these variables are both positively correlated with the initial concentration of nitrate in the medium. This relationship was further examined by calculating correlation coefficients between the two variables within 40 of the individual treatments from the two main experiments; the treatments covered a wide range of initial nitrate concentration in the medium. A significant correlation would indicate that nodule number depends on the time of initial nodulation, as suggested by Nutman (1949) from his delayed-inoculation experiments. However, in the experiments being discussed only 2 of the 40 correlation coefficients were significant at the 5 per cent. level, indicating that the effect of nitrate on nodule number is independent of the effect of nitrate on the time of initial nodulation. This conclusion is supported by the observation that all forms of combined nitrogen supplied to the plants increased nodule number (Table 3), whereas only nitrate and nitrite nitrogen delayed initial nodulation.

In Expt. 1 the agar-type and preinoculation treatments influenced the time of initial nodulation and these treatments interacted with the other treatments in the experiment (Table 2). Shredded agar stimulates the initial nodulation of the clovers, particularly when the medium is prepared with tap water. The complexity of the effect of shredded agar on the time of initial nodulation can be seen from the observations made on the inoculated preplants of Expt. 1. In conjunction with this phase of the work, an experiment was also carried out with the same types of medium prepared with Jensen's instead of Thornton's nutrient solution, and sown with lucerne or red or white clover inoculated with appropriate strains of rhizobia. Table 6 shows the results with the clovers.

On Thornton's medium, the clovers in the Bacto-agar/tap-water treatments nodulated later than in the Shredded-agar/tap-water treatments, whereas on Jensen's medium they nodulated earlier. The reason for this effect on the clovers has not been determined, and the effect of the inorganic nutrient composition of the medium needs examining before any conclusion can be drawn. In this experiment agar-type did not affect the initial nodulation of lucerne.

TABLE 6

The Effect of Medium-Type on the Time of Initial Nodulation, in Days, of Red Clover and White Clover. 10 replicates per mean

		Red clover		White clover	
Water	Agar	Thornton	Jensen	Thornton	Jensen
Distilled	Bacto	9·8	9·2	10·1	9·9
	Shredded	9·2	9·7	10·6	11·6
Tap	Bacto	12·5	10·7	16·8	11·4
	Shredded	10·8	11·9	13·3	13·1

Difference between means necessary for significance: 2·2 d. ($P = 0·05$.)

The effect of preinoculation is also difficult to determine and interpret. In fourteen of a possible twenty-four comparisons between preinoculated and control treatments in Table 2, the preinoculation means are earlier by at least 0·5 day than control means (in nine treatments the difference is 1·0 day or more). This effect seems not to be associated with preplanting, agar-type, water-type or species. In further experiments these differences were usually but not always greatest with lucerne.

Although the removal of nitrate by preplanting accounts for nearly all the effects reported in this paper, some experiments done on medium prepared with Bacto-agar, distilled water, and Thornton's modified nutrient solution suggested that nodule initiation may sometimes be stimulated; see Table 1, Expt. 2 (Fig. 1), and Gibson (1959). There is evidence from other experiments (Gibson, 1959) which indicates that the inorganic nutrient composition of the medium, the environmental conditions, and the bacterial strain are important in determining the degree of stimulation, and until they are better understood further discussion of a small preplanting stimulation, which may depend on root secretion, should be deferred.

Although the plants grown on preplanted medium in Expt. 1 formed the same number of nodules as the controls, in other experiments (Table 3) preplanting a N-free medium lowered the number of nodules on the test plants by 20–50 per cent. These observations agree with Nutman's hypothesis (Nutman, 1945, 1952*a*, 1953) that the roots and the nodules secrete something that retards nodule formation. Recent observations by Kefford, Zwar and Brockwell (personal communication) indicate that this is probably an auxin-type compound.

SUMMARY

1. Nodules are initiated earlier on clover and lucerne plants grown on a medium prepared with distilled water than in the same medium prepared with tap water containing about 6 p.p.m. nitrate nitrogen.

2. Preplanting (growing a preliminary batch of seedlings) hastens nodule initiation on a tap-water medium, because the preplant removes nitrate. The hastening effect of adding charcoal to tap-water medium may be similarly explained by adsorption of nitrate.

3. Nitrate and nitrite ions at very low levels (20 μ gm. N per seedling) delay nodulation; ammonium sulphate, asparagine, or urea do not delay nodulation, although they are assimilated at about the same rate as nitrate by the young seedling.

4. Although nitrate at low concentration delays nodule initiation it increases the rate nodules are formed, which partly explains why final nodule numbers are decreased by preplanting the tap-water medium.

5. Preplanting sometimes hastens nodulation slightly even when the medium is free from nitrate. This appears to be affected by type of agar, inorganic composition of medium and time of inoculation, and these influences should be examined before root secretions need be invoked.

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The Stability of the Growth Pattern of Young Apple-trees under Four Levels of Illumination

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With four Figures in the Text

ABSTRACT

As part of a programme on the characterization of the growth pattern of the apple-tree, uniform 1-year-old plants carrying two new shoots were grown for a season under 100, 78, 41, or 24 per cent. natural light. Weights of leaf, new stem, old stem, and root were determined by sampling on 6 occasions. The dry-weight increments over the whole season ranged from 17 to 151 g. of which, in full light, 22 per cent. was leaf and 22 per cent. was root; in deep shade, 32 per cent. was leaf and 8 per cent. was root; and under all conditions 56–60 per cent. was stem. These percentages were the cumulative result of current rates of growth of leaf, stem, and root over the whole growing period. The rates were not constant relative to one another, but the much greater quantity of growth made over the period 100–200 days from bud-break swamped earlier differences. All treatments showed the same basic pattern of growth with minor differences increasing with the intensity of shading. These differences are discussed in relation to utilization of reserves and the demands of the various regions of the plant. The response to shading was analysed into the effects of reduced light interception, increased leaf dispersion, increased leaf proportion, reduced length of growing season, and lighter leaf weight at the beginning of the 20-leaf stage. The result of these factors was a doubling of the growth expected in the absence of response. It was concluded that the pattern of growth is stable, and the conditions for the perpetuation of such stable patterns as a necessary qualification for tree growth are briefly discussed.

INTRODUCTION

AMONG the consequences of becoming a tree is that one year's growth remains to influence the growth in succeeding years. Unlike the situation in annuals and herbaceous perennials, the accumulated past-growth forms the plant body at the beginning of each year's growth, and is the site for the deposition of much of the current year's secondary thickening. Trees, therefore, must have one qualification not essential to other plants if they are to grow, for while other plants have only to maintain sufficient balance between the growth of the parts to permit the formation of seed or propagules before the end of the growing season, in trees the woody surfaces represent a demand for anabolites that would seem potentially capable of swallowing up most of the current assimilate, thus preventing the establishment of the characteristic repetitive pattern of growth. Since this does not occur, there must be some sensitive yet strong regulator controlling the relative growth of the parts. A number of experiments on young trees, especially fruit-trees, have confirmed

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that the annual growth is distributed within fairly narrow limits between leaves, stem, and root (Hatton *et al.*, 1924; Vyvyan, 1934; Cameron, 1939; Goodall, 1955; Maggs, 1959, 1960 *a*, *b*), and as a first step towards the analysis of the regulatory mechanism its response to various environmental factors must be determined. The present paper is intended as a contribution towards the solution of the problem.

There are, moreover, important economic implications in the control of the distribution of increment, for what is not utilized is waste. As Watson has pointed out (1959), the yields of some crops are now approaching their theoretical maxima under current methods of production, in which case the yield of economic end-product may be increased by reducing the proportion of unwanted by-product. This approach is often implicit in breeding programmes, manurial trials, &c., but quite understandably, the yield of end-product is taken as the main aim. In many cases the factors controlling development have been studied and exploited by reducing the time from planting the crop to harvesting, but it is only incidentally that the production of unwanted material has been reduced. In fruit-trees an investigation of the possibilities of reducing unwanted by-product would, if successful, have especial value. Part of the annual dry-matter production is cut away each year as prunings, the branches and trunk are often much stronger and thicker than is required by the stresses they have to resist, while a heavy crop seriously competes with the other growth processes of the tree (Wilcox, 1937; Barlow and Preston, 1960).

For apple-trees, the factors which have been shown to affect the increment and its distribution include scion variety (Amos *et al.*, 1931; Vyvyan, 1931*a*), soil type (Rogers and Vyvyan, 1934), magnesium status (Delap and Ford, 1958), and pruning (Maggs, 1959). Light (Heinike and Childers, 1937), soil moisture (Rogers, 1956), and nitrogen (Ruck and Bolas, 1956) have been shown to affect increment, and judging from their effects on other plants, are almost certain to have affected its distribution. Temperature effects do not appear to have been adequately studied. Moreover, apart from Vyvyan's account of the Net Assimilation Rate (1957), no attempt appears to have been made to follow changes in the distribution of increment throughout the growing season.

In the experiment described below light was chosen as the experimental variable for several reasons. The provision of a range of intensities was within the resources available; large effects on the growth increment and compensatory changes in its distribution could be anticipated, and sufficient numbers of trees could be grown experimentally to permit replicated sampling at intervals. The present paper is concerned mainly with changes in weight and its distribution; a subsequent one dealing with carbohydrate utilization will be presented by C. A. Priestley, who shared equally in the experiment.

MATERIALS AND METHOD

Materials. Stoolbed shoots of the rootstock variety MM. 106 were carefully graded for length, diameter, freedom from side shoots, and damage. They were then assigned to weight classes rising in steps of 0·5 g. After discarding classes heavier than 15 g. or lighter than 8 g. the remaining classes were aggregated to give 8 classes each containing about 30 plants. Replicate sets for all 6 sampling dates were taken from within each class, thus confounding size and block effects. The trees were then pruned to half their length to ensure an adequate and constant proportion of new shoot within each treatment. Twenty-four replicates were potted, 6 sets (48 plants) going to each of the 4 plots. Three sets of replacement trees were put into '3-inch' (8-cm.) pots, and samples were taken for various analyses. The experimental trees were grown in '10-inch' (25-cm.) pots containing John Innes compost without added lime, spaced in a double line along the plots. Each pot held 7 litres of soil. After bud-break but before stem-elongation began, all but the two top shoots were rubbed off each plant.

Mildew (*Podosphaera leucotricha*) was controlled by frequent sulphur dusts whenever seen. From mid-July onwards 001 ml. Hoagland's nutrient solution (Hoagland and Arnon, 1938) was added weekly to each plant. Water was given according to standard management; the heavily shaded trees dried out more slowly and in periods of rain remained moist longer than those in full light.

Site. The experimental area was screened from early and late sun by low buildings, and was divided into four plots each 24 ft. (7·4 m.) long and 4 ft. (*c.* 1·2 m.) wide separated by alleys of the same width. Four levels of shading were obtained by surrounding the plots by cages topped with variously spaced lathing and hung with coarse netting (hop lewing) down the sides. Measurements of light intensity were made with a pair of balanced photocells and a galvanometer. The percentage light intensity relative to the unshaded plot averaged over the day and during the season was—Plot 1 (unshaded), 100; Plot 2 (light shade), 78; Plot 3 (medium shade), 41; Plot 4 (dense shade), 24.

Experimental procedure. The effect of different levels of lighting upon growth was determined by weighing 6 samples of plants during the season. Previous experiments had shown how various stages of growth might be recognized, and the stages at which the samples were taken are summarized as follows:

Sample	*Description*	*Development stage*
1	An initial sample at the beginning of bud-break	Bud scales separating
2	Minimum weight, previous growth at expense of reserves	Leaves expanded, new stem just visible
3	Onset of period of constant growth-rate	10 expanded leaves
4	Middle of period	20 expanded leaves
5	End of period of constant growth-rate	Cessation of internode elongation
6	Leaf fall	Terminal rosette of leaves only remaining

The depletion of reserves in early growth was an especially interesting phase of carbohydrate utilization, and on the assumption that the experimental treatments had little effect between stages 1 and 2, sample 1 was spread over four sub-stages—1/1, dormant; 1/2, bud-scales separating; 1/3, small leaves expanded; 1/4, rosette of leaves but no stem visible. Two trees were taken from each plot for each sub-stage. Trees were nominated to each sampling at the beginning of the experiment and harvested as they reached the required stage, and it thus happened that, owing to variation in bud-break, some trees for stage 3, for example, were harvested before some in stage 1. To reduce extraneous variation, trees still dormant by June were discarded and replaced by spares. This led to some deficiencies in stage 3 as the spares were then past that stage of development. Records were kept of the development of each tree until it was harvested.

In analysing the trees, leaves were stripped in the field and after washing off the soil, the roots were blotted dry and a check total fresh weight taken. The roots were cut off, weighed, and sampled by slicing through a tight bundle of roots. New stems were cut off, measured, and weighed; samples were taken by cutting the stems into six equal lengths and sub-sampling from each of these; bark and wood were separated; the diameter of each shoot was measured 5 cm. above its base. Old stem was separated into above and below ground portions, and the diameter measured before and after stripping off the bark; sub-samples were separated into bark and wood. Before surplus material was discarded, the sum of the weights of the parts was checked against the check fresh weight. Samples were oven-dried at 95° C., and abnormal values of dry wt./fresh wt. ratios checked carefully.

RESULTS

The argument behind the experiment was that the response to shading would give a measure of the tree's plasticity and could be used to gauge how far the distribution of the increment to the various parts of the tree was fixed in a stable pattern. The total growth must be analysed into its components in time (stages) and space (parts), the modifications due to shading assessed, and the resultant effect of each modification on the final end-of-season growth calculated. The main effects of shading pertinent to this analysis appeared to be on bud-break, affecting the length of the effective growing season, on rate of leaf production, leaf-size and leaf thickness, affecting the amount and duration of the assimilating surface, and on the relative amounts of leaf, root, and stem, affecting among other things the assimilating, absorbing, and cambial surfaces.

Development

A scale of bud development has been found convenient for measuring the early stages of growth, and was used in this experiment up to July 2. This scale ranged from 1 for dormant buds to 6 when over 5 cm. of new stem was visible. In average conditions the scale is approximately linear in time, but where bud-break was considerably delayed, the early stages were accelerated by the warmer conditions. Buds on the majority of trees began to break by the end of April, although 8 per cent. of the trees were still dormant by June 1. Because of the weighting effect of persistently dormant trees, treatment differences in this early phase of growth are better shown by a frequency diagram than by averages. Fig. 1 shows the distributions of the averages of the five weekly observations between April 16 and May 14. The data suggest that light shade did not affect the rate of early growth, while dense shade slowed it down. There is also some evidence that the population consisted of two groups differing in their readiness to start into growth.

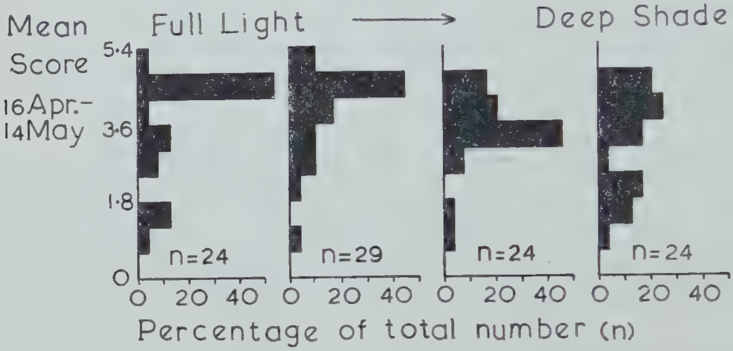

Fig. 1. Frequency distribution of scores for bud development. The scale ranged from 1 for dormant buds to 6 for those with new stem between 5 and 10 cm. long. The data summarized are the means of 5 scores obtained between April 16 and May 14, 1958.

Stem Length and Leaf Number

Of the two shoots allowed to develop, the shorter (usually the lower) was most retarded by shading. When stem elongation ceased, the longer shoot averaged 110 cm. in full light, 103 in light shade, 99 in medium shade, and 76 cm. in deep shade, ±6·6 cm., and the shorter shoot 75, 50, 32, and 3 cm. The differences between the longer shoots were attained within the first 70 days of stem elongation. Leaf production was closely linked with stem elongation; the number of leaves eventually produced was 47, 44, 45, 36, ±2·6 on the longer shoot, and 38, 25, 18, 5 on the shorter, while the corresponding average internode lengths were 2·34, 2·32, 2·25, 2·08, ±0·08, and 1·96, 1·42, 1·42, 0·99 cm. Between the 15th and 50th days from bud-break, the rate of leaf production was 1 leaf per week faster in full light than in deep shade, and from the 50th to the 160th day it was 0·4 leaves per week faster.

In these respects the effect of shading was drastic only when dense. By contrast, even a light degree of shading greatly reduced the number of leaves on secondary shoots, so that averages of 31, 7, 1, 0 secondary leaves per plant were found at the 5th sampling (October).

Leaf Size and Shape

When leaf production ceased, the leaves on the longer shoot averaged 44, 58, 55, 43, ±2·7 cm.^{2} in area, and on the shorter 36, 43, 34, — cm.^{2}, the differences having been established by the time the plants had 20 leaves. Earlier on, however, at the 10-leaf stage, when a greater proportion of the leaves were still expanding, the heavily shaded leaves were the largest (8·8, 7·2, 9·2, 10·9 cm.^{2}/leaf). This indication that heavy shade increases the early expansion of the leaf is supported by measurements on individual leaves made in August. Then the first leaf separated from the central spear averaged 4·9, 4·3, 3·2, 5·5, ±0·46 cm.^{2} compared with 45 6, 58·6, 57·6, 45·9, ±2·48 cm.^{2} for the fully expanded leaves lower down the shoot.

The form of the leaf was changed by shading, since the average leaf weight steadily decreased (0·66, 0·64, 0·43, 0·17 gm./leaf) while the length/breadth ratio of samples of 5 leaves per plant averaged 1·46, 1·58, 1·54, 1·47, ±0·04. Microscopical examination of leaf sections showed that the increase in specific area (area/g.) induced by shading was due to both an increase in the internal gas space of the leaf and a decrease in lamina thickness. In medium shade even the uppermost palisade cells were separated from one another for a quarter of their length and in deep shade for two-thirds of their length. The spongy mesophyll was also more dispersed. Although the evidence in Haberlandt (1914) is that shading decreases the depth of the individual palisade cells, in this experiment it was increased. This may be due to compensation for a decrease in the number of clearly defined palisade layers from about two to one.

Total Leaf Area

At first, total leaf area increased relatively faster than leaf number; subsequently it increased more slowly. This was because the leaves produced at the beginning and end of the growing season were smaller than the others. Treatment differences, too, were soon apparent. The depressing effect of shade on the rate of leaf production was greater than any increase due to larger individual leaves, with the result that trees in full light always averaged the greatest leaf area, closely followed by the lightly shaded trees. The medium-shaded trees differed little from those lightly shaded until 70 days from bud-break; thereafter expansion was slower and by October their leaf area was some 700 cm.^{2} less. The heavily shaded trees developed more slowly from the beginning of leaf expansion and finished growth with about one-third of the area of the trees in full light. On October 10 the treatments averaged 2,900, 2,800, 2,100, and 1,100, ±235cm.^{2} leaf per plant. These figures include shed leaves.

Stem Taper

Contrary to the findings of Auchter and Schrader (1926), deep shade decreased stem length by 30 per cent. and internode length by 11 per cent. However, the relation between length and basal diameter was found to be

$L = bD^{1.14}$, where the values of b increased from 0·22 in full light, through 0·24 and 0·30 to 0·36 in deep shade. Thus, had the stems been of equal diameter, those in deep shade would have been nearly twice as long as those in full light, so that the tendency has in effect been towards the 'normal' etiolation pattern.

Weights

Data on bud-break stages and leaf areas indicated that some of the harvest values needed adjustment *within* treatments to give a more accurate picture of the time-course of weight changes. The adjusted data are plotted against time in Fig. 2. Analyses of variance of the logarithms of the increments from planting to sampling showed that treatment differences were highly significant after sample 2.

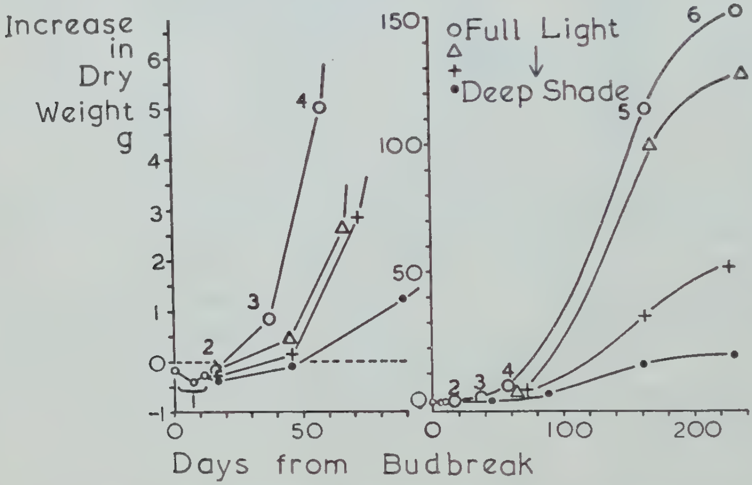

FIG. 2. Time-course of increase in dry weight. Numbers in the graphs indicate sampling stages. The figure on the left shows the earlier samples in greater detail.

Attention is drawn to the following points:

1. The slight loss in weight in early growth, and the length of time required by heavily shaded trees to regain it.
2. The early appearance and small initial magnitude of treatment differences.
3. The near-constancy of the growth-rate over the period of rapid growth.
4. The rapid increase in treatment differences in this phase of rapid growth.
5. The continued increase in weight after the cessation of terminal growth.
6. The wide range of increment.

The maximum rate of loss in weight occurred between bud-break and the unrolling of the leaves; it subsequently declined. It is most unlikely that this decline was due to a reduced respiration rate, and it must be concluded that appreciable photosynthesis occurred as soon as the leaves were exposed.

Root growth and stem thickening continued after the apical growth had stopped; the data suggest that it continued almost unchecked right up to leaf-fall. Heinicke (1935) showed that young trees with but a tuft of leaves remaining in the autumn produced 9 g. of new roots compared with 2 g. produced by similar leafless trees. Vyvyan (1931*b*) and Hammerle (1901) have noted autumnal root growth. Nevertheless, in these apple-trees, the increment made in the month after cessation of extension growth was about half of that made previously in the same growing season, strongly suggesting that in these conditions cessation of apical growth imposed little limitation on the growth of the rest of the tree.

Proportional Distribution of Increment

By the end of the growing season, the distribution of increment between leaf, stem, and root acquired a characteristic pattern varied by treatment only in the case of leaf and root. The distributions for the last two samplings are given in Table 1.

TABLE 1

Percentage Distribution of Increment in Dry Weight from Planting to Sampling

	12 Oct. 1958					14 Dec. 1958				
Shade	Nil	Light	Medium	Dense	*s.e.*	Nil	Light	Medium	Dense	*s.e.*
Leaf . . .	30	33	36	42	*1·6*^{xxx}	22	23	30	33	*1·1*^{xxx}
New stem . .	41	39	39	40	*1·0*	40	43	43	43	*1·1*
Old stem increment	14	15	14	11	*0·6*^{xx}	16	17	17	16	*0·6*
Root . . .	15	13	11	7	*0·7*^{xxx}	22	17	10	8	*0·7*^{xxx}

On each occasion, the leaf and root percentage varied reciprocally, the leaf increasing by about 10 per cent. and the root declining by the same amount as shading increased. The total stem, therefore, remained fairly constant, with little change in the proportions of either new-stem or of old-stem increment. Between October and December, over all treatments, the percentage of leaf declined by about 10 per cent., the corresponding increase being spread over all the other parts. This was due to the continued assimilation by a constant or even declining quantity of foliage.

The data available permit the estimation of the distribution of increment during the course of growth. In early growth, when new growth and respiratory losses in the absence of assimilation combined to produce negative or zero increments, a special method of computation was employed. This consisted in using 'turn-over' for 'increment', as shown in the following example. Consider a storage tissue sending out new growths, prior to effective photosynthesis. If, neglecting respiration, the old tissue loses x g. weight and the new tissues therefore weigh x g., the overall change in weight is zero, and no meaningful calculation can be made on the distribution of increment. The turnover in material, however, is $2x$, and of this 50 per cent. has been derived from loss from old tissue, and 50 per cent. gain to new tissue. Where there has been some assimilation, and the new tissues now weigh $x+a = b$, the turnover is $2x+a$ or $x+b$. With further growth and an increase in the weight of the storage organ, the method breaks down as x becomes unknown, but by this time the total increment is sufficiently large for the distribution to be calculated as proportions of total increment without these finer points much affecting the result. In practice the values were calculated by ignoring the sign of any changes in weight of the parts from sample to sample in calculating turnover, but retaining the sign to indicate gain or loss (as in Fig. 3).

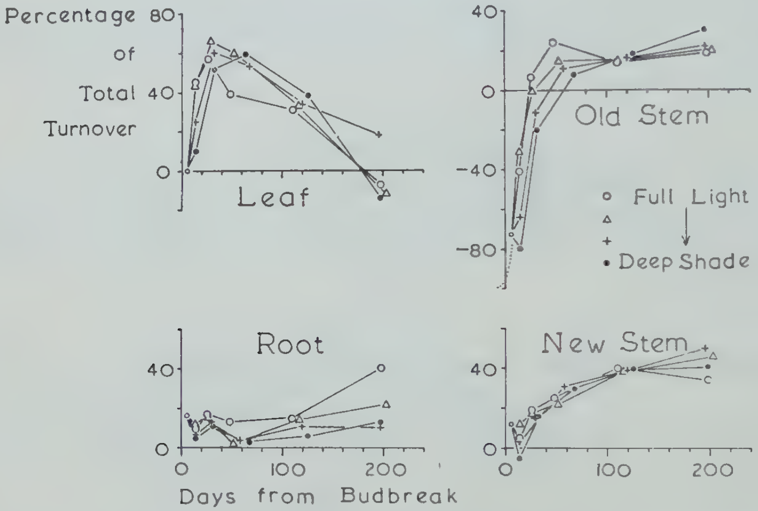

FIG. 3. Percentage distribution as leaf, new stem, old-stem increment, and root of increments between sampling occasions. Where loss in weight is shown, values are based on total turnover, i.e. arithmetical sum of changes in weight of the parts. Dotted line in 'Old Stem' figure represents change-over from initial period when no new tissues are forming and all the loss in weight is respiratory.

Respiratory losses in the early stages will be discussed in a subsequent paper by C. A. Priestley. The changes in distribution *from sample to sample* are shown in Fig. 3. In spite of each point containing the errors from two samplings, the curves appear reasonably consistent. The general similarity of the effects of the treatments is remarkable, and differences can usually be reasonably interpreted in physiological terms. The period during which the old stem lost weight by export to respiration lasted until 30–80 days after bud-break according to treatment. When the old stem loss comprised the whole of the turnover (−100 per cent.), this loss was all respiratory wastage (dotted line in Fig. 3, Old Stem). As synthesis of new tissues began, this negative percentage decreased numerically and became positive when deposition of new material was greater than the current wastage of the old stem. This break-even point was delayed by shading up to fifty days. So far as could be detected loss in weight of the old stem was not increased by shade during this period, so that the delay in making up the initial loss was due to a lower rate of deposition of new material rather than to a continued export of material from the old stem. Leaf tissue was the principal product of synthesis in early growth, but between the 10- and 20-leaf stages it declined in proportion in favour of the stem tissues. As all leaves were collected and dried the morning after they were shed, the decline to negative values at the end of the season probably represented an actual export of material above current photosynthesis from the leaf as much as wastage of tissue by attrition and experimental loss. The effect of shade was to delay the peak utilization of material as leaf.

All treatments showed a parallel course in the utilization as root. Very few new roots were ever seen until the bud scales were appreciably separated[1] and at this stage they represented all the new growth, but once leaf expansion began the old stem lost material to both root and shoot, and the root percentage of the turnover declined until stem elongation was well under way. The percentage then increased concurrently with shoot development to the 10-leaf stage, and again declined to an ill-defined minimum about the 20-leaf stage. This first rise followed by a decline possibly corresponded with the establishment of continuity of the current season's secondary thickening between leaf and root, the subsequent decline being due to the greater development in the stem than in the root at this stage. Subsequently, secondary thickening and lignification in the root were determined by the surplus of material permitted by the treatments, so that in full light the increment added after the end of extension growth consisted to a much greater extent of root than it did in shade.

Treatments had little effect on the distribution of the increment over the period from the 20-leaf stage to the cessation of growth, i.e. during the phase of constant growth-rate. The values for new-stem and old-stem increment were remarkably constant, and leaf and root varied reciprocally. As the quantity of increment made during this period was between ten and twenty times that made previously, earlier differences in distribution have been swamped in the cumulative figures given in Table 2. The considerable increment made subsequently until leaf-fall tended to be distributed similarly, but somewhat less regularly. It would seem that for certain kinds of treatment, sampling at the end of extension growth would give a more critical picture of the distribution than sampling at leaf-fall or later.

Relation between Stem Thickening and Total Increment

In a recent pruning experiment, Maggs (1959) found a close relation between cambial activity and total growth, resulting in a linear regression between the *Increase in Weight per Centimetre Length of the Old Stem* and the *Total Increment*. The wide range of increments in the present experiment permitted a further check on this relation. *Total Increment in Dry Weight* was linearly related to the *Increase in Weight per Centimetre Length* and also to the closely analogous *Increase in Stem Cross-section Area*. A common relation held for all treatments (Fig. 4). The first of these stem measurements may only be obtained from cut-up trees, but the second may be obtained by caliper or girth measurements; a statistical comparison of the two relations showed that both were about equally efficient in estimating increment, the S.E. of the regression coefficient being in each case approximately 3 per cent. The reversed positions of the sets of points (o and +) in the two graphs may be interpreted as due to the accumulation of reserve materials between October and December with little increase in stem diameter.

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[1] This is not always the case.

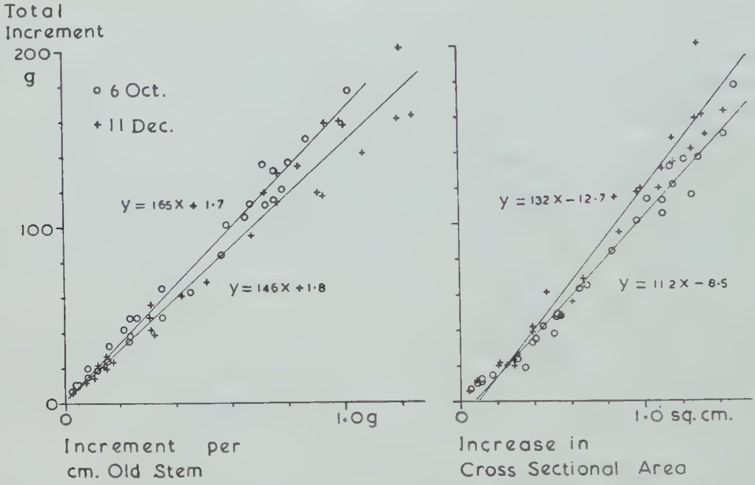

FIG. 4. Relation on two occasions between *increment per cm. length old stem* and *total increment*, and between the *increase in area of cross-section* and *total increment*.

To test whether this linearity between stem thickening and total increment over all the treatments reflected a uniformity in the stem anatomy, cross-sections of the old stem were examined. Vessel density, vessel diameter tangentially and radially, and depth of lignification were measured. It was concluded that shading had little effect on the pattern of anatomical differentiation in the stem, but delayed the rate at which the trees followed the pattern. The formation of lignin in the thickened fibres and vessels was markedly delayed by dense shade.

DISCUSSION

Effectiveness of the Adjustments to Shading

Although the responses of plants to shading have often been described, few attempts have been made to assess the contributions of the various modifications to the changes in total increment (Blackman and Black, 1959). In Table 2 are presented the results of calculations as to the effects of several factors had they operated in full light to the same extent as they did in shade, all the other factors operating at full-light values. The argument of Gregory (1918) and Briggs, *et al.* (1920) can be adapted to provide an analytical model. During a period in which the leaves, stems, and root are utilizing fairly constant fractions of the total increment, the average production of increment

TABLE 2

Analysis of Growth. 20-leaf Stage to the End of Extension Growth

Item		Absolute values in full light	Relative values, full light = 100			
			Full light	Light shade	Medium shade	Deep shade
1	Light intensity	?	100	78	41	24
2	Total increment, g.	107	100	97	27	12
3	N.A.R. mg./cm.^{2}/day	0·977	100	85	39	25
4	Specific leaf area cm.^{2}/g.	85·0	100	124	178	299
	Theoretical effect of single factors on increment					
5	Light (l)		100	52	14	6
6	N.A.R. (a)		100	64	14	7
7	Specific leaf area (s)		100	205	950	20,700
8	Leaf proportion (p)		100	112	115	152
9	Length of growing season (t)		100	87	72	38
10	Initial leaf weight (Lo)		100	79	81	45

may be considered to be divided into a part, p, of leaf, and $1-p$ of non-leaf. The part p then functions to produce increment at a rate a' g./g. leaf. a' may be further analysed into the increment/cm.^{2} leaf a, and the leaf thickness or area/g. leaf s. If at the beginning of the period the tree has a weight of leaf, L, the increment over a unit period is asL of which $pasL$ is leaf. The next period therefore starts with leaf $L(1+pas)$, produces new material $asL(1+pas)$, so that by the end of a series of periods the total leaf weight is $L(1+pas)^t$, and the increment is

$$asL+as\,L(1+pas)+asL(1+pas)^2 ... +asL(1+pas)^{t-1} = \tfrac{L}{p}[(1+pas)^t-1].$$

When t is measured in periods sufficiently short for a to become very small, a becomes identical with the Net Assimilation Rate, defined as

$$E = \frac{W_2-W_1}{L_2-L_1}\times\frac{\log_e L_2-\log_e L_1}{t_2-t_1},$$

over similar periods. In the present case, when t is measured in days, a differed from the N.A.R. by 3–5 per cent. The present formula is, however, more easily used in this analysis.

There are considerable assumptions and limitations in this model. The value of a (or of the N.A.R.) is the average value over the whole interval, irrespective of ageing effects and of the differential effects of the treatments on shorter-term fluctuations (Vyvyan, 1957; Heinicke and Childers, 1937). It is also implicit that the increment increases nearly exponentially with time, so that where this is not so the importance of the pas and t factors may be distorted. Changes in day-length have also been ignored. A further assumption is that all forms of the increment (leaves, stem, and root) are equivalent in terms of weight; that is, that 10 g. of leaf are the resultant of the same amount of photosynthesis as 10 g. of root; and that the relative levels of growth promoters and inhibitors are not altered. These limitations seem sufficient to preclude comparisons between different stages of growth, but in view of the general correspondence between the pattern of growth of the different treatments, it seems reasonable to compare the treatments over the period when growth is proceeding fairly steadily, i.e. the period between the 20-leaf stage and the end of growth.

This comparison has been made in Table 2. The extent of the response to shading may be seen by comparing the relative increments (item 2) with those obtained by assuming that the N.A.R. was proportional to light intensity without change in form (item 5). It appears that the young apple-tree's response to shade is sufficient to double its 'expected' increment, irrespective of the degree of shading. It must be presumed that this response falls off in shade lighter than that used in this experiment. It is also evident that the N.A.R. (item 3) is closely proportional to the light intensity (item 1), suggesting that shading has not impaired the leaf's capacity for photosynthesis.

The response is the resultant of both compensatory and penalizing modifications. The most potent positive modification was the greater dispersion of the leaf tissues; this increased threefold as the shade increased (item 4) and in full light would have been sufficient to have produced a 200-fold increase in growth within the conditions of the equation (item 7). The other positive factor was the increase in leaf proportion (item 8). Although by no means so powerful an adjustment, nevertheless it is apparent that a small change in leaf percentage is capable of producing significant effects over a period. The negative deleterious responses were the consequences of the slower rate of bud-break and development. Since extension growth ceased nearly simultaneously for all treatments, the delay in bud-break (about 10 days) and the longer time to reach the 20-leaf stage (about 20 days) reduced the effective growing period in deep shade by 30 per cent. According to the formula this reduced the increment by 60 per cent. (item 9). This must be an over-estimate, since the data of Fig. 2 show a nearly linear increase with time over the period. The lower initial leaf weight, resulting from a reduced rate of production and from the suppression, more or less, of the second shoot, reduced growth by up to 55 per cent. The effect of the suppression of the second shoot has not been brought out separately by the foregoing analysis. In deep shade the second shoot never grew more than a few centimetres, and the plants were deprived of half their potential production of leaves. Had all the plants been limited to a single shoot, the increment in full light would probably have been reduced by between 20 and 40 per cent. (Maggs, 1960*a*). On the other hand, had there been no reduction of shoot number it is probable that the differences would have been even greater.

In the orchard it is probable that shading of the lower and interior parts of the tree by the upper and peripheral foliage may reduce growth, as well as impairing the development of fruit colour. Thus Christopher (1934) found that the light intensity at a single spot within the tree varied widely during the course of the day, and Westwood and Gerber (1958) have shown that the light inside the crown of heavily pruned peach-trees may be reduced during the growing season to one-fifth of the outside light, while in unpruned trees, because the weight of the fruit and foliage pulls the branches down, the interior light intensity may actually increase as the season advances. In view of the marked effects of shading on the growth of the apple-tree, it is clear that the contribution of shading to the effects of various pruning régimes merits further study.

The Distribution of Increment

The main object of this experiment was to see how far the normal pattern of distribution of increment could be distorted by shading. A comparison of Figs. 2 and 3 shows how small was the effect of shade on distribution compared with the enormous effect on amount. Particularly was this so in the case of stem regions; the increment in new stem plus old stem varied by no more than 4 per cent. of the total; while even leaf and root only varied by 12–14 per cent. These changes are small compared with the nearly 10-fold range in increment in weight. Certainly there are differences between the treatments in Fig. 3; but the significant point here is, for example, that while the new-stem increment in deep shade was only 7 g., it was built up on a time pattern very similar to that for the 60 g. increment in full light, and not by a reiteration of, say, the first quarter of it.

Stem percentage has now been shown to vary very little in response to light, pruning (Maggs, 1959), shoot number (1960*a*), water status, and the complex of factors by which the inside of a greenhouse differs from the outside (1960*b*). If these results, obtained on young non-cropping trees, are applicable to the fruiting trees, it would seem that pending another line of attack, a considerable part of the tree's production of dry matter cannot be utilized commercially. Against this, however, it may be noted that Chandler (1925, 1934) has presented evidence suggesting that the presence of a crop enhances the productivity of the leaves, so that growth and cropping may not be completely alternative.

The Perpetuation of Pattern

In the introduction to this paper, it was postulated that the constitution of a tree must include some mechanism capable of balancing the growth of its various regions. Such a mechanism has now been demonstrated operating over a wide range of increments for a single season. A repetition of the pattern over two seasons has also been found in an experiment with varying numbers of primary shoots (Maggs, 1960*a*). If it be assumed that similar relations apply from year to year over the life of the tree, then the various limiting sizes of various varieties on various sites may be attributed to some drift with time in the characteristics of the control. Possibly to define a mechanism by so broad a group of results will lead to confusion between a multiplicity of causes, but the manifold effects of IAA show that the reverse is also liable to occur. Such a mechanism should be distinguished from growth correlations, referring rather to the interaction between specific organs, whereas the present concept refers to regions and the collective increment in leaf, root, &c. It has some affinity with physiological homeostasis maintaining constant the animal's internal environment, but in our case the constancy refers to increment rather than to the existing structures. Lerner (1954) has, however, used genetic homeostasis in the sense of restoring and maintaining the genetic structure of a population, and this must be largely due to the activity of the population increment. Again there are affinities on the one hand with Net Assimilation Rate, but free from that ratio's dependence upon leaf proportion, and on the other with the allometric equation, but with the time factor well in the picture.

There are several mechanisms which are probably involved in the perpetuation of pattern—hormonal, nutritional, water-supply—and the list is not comprehensive or exclusive—but I know of no specific demonstration of the action of any of them. The close relation between stem-thickening and total increment (Fig. 4) suggests that the cambium is active to the degree permitted by the passage of substrates from xylem and phloem, so that competition between regions is a further potential factor. Duff and Nolan (1953), from a consideration of the distribution of the annual thickening, in time and position, on the trunk of *Pinus resinosa*, concluded that the controlling internal factors were nutritional gradients determined by the placing and illumination of the foliage. A non-diurnal decrease in sugars, and in particular sucrose down the trunk, has been reported by Dixon and Atkins (1916), Harris *et al.* (1917), Huber *et al.* (1937), and Zimmerman (1958). One may speculate whether this gradient is the result of utilization by the cambium, whatever its other consequences. It is apparent that the subject at this stage is a sort of Elysian Field populated by spectral possibilities from amongst whom the few actualities must be enticed by cunning stratagems in the shape of further experiment.

SUMMARY

Rooted 1-year plants of uniform size of the apple rootstock MM. 106 were grown with two shoots for one season in large pots under four levels of shading. The shade averaged 100, 78, 41, and 24 per cent. of natural daylight. Six samples were taken during the course of growth.

2. Shading reduced length of new stem especially of the second shoot, number of leaves, internode length, and total leaf area; individual leaves had the greatest area in intermediate shade and the greatest weight in full light; these changes were due to an increase in the internal air space and to a reduction in leaf thickness.

3. Total leaf area was reduced by shading, the reduction from full light to light shade being small compared with the reduction from light shade to medium and dense shade.

4. Total dry weight increased approximately linearly over the interval 60 to 200 days from bud-break. The increment was reduced by shading, the values to December 6 being 151, 127, 52, and 17 g. Treatment differences were statistically significant from the 10-leaf stage, and were apparent soon after bud-break (Fig. 2, Table 1).

5. The distribution of increment to leaf, new stem, old stem, and root followed a similar but not identical pattern under all treatments. These are shown in Fig. 3. The period of export from the old stem but not the total deficit was increased by shading. The result of these developments was that, of the total increment over the season, new-stem and old-stem increment each comprised a nearly constant proportion under all treatments, while the leaf proportion increased with shade and the root proportion decreased (Table 2).

6. Over all treatments, total increment was closely proportional to the increase in weight/cm. length of the old stem and also to the increase in cross-sectional area at the mid-point of the old stem (Fig. 4).

7. Over the period between the 20-leaf stage and the cessation of growth, the responses to all levels of shading were sufficient to double the increment expected in the absence of response. Of the two compensatory responses analysed, the increased dispersion of the leafage was much more efficacious than the increase in the proportion of leafage. The two penalizing responses—shorter effective growing season and lower leaf weight at the beginning of the 20-leaf stage—were equally deleterious in their effects (Table 2).

8. It is concluded that a considerable proportion of the tree's production of dry matter cannot be used commercially because of the inflexibility of the growth pattern. Some conditions for the perpetuation from year to year of such a pattern are discussed.

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Studies on the Membrane of the Almond Endosperm Haustorium

BY

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With six Plates and one Figure in the Text

ABSTRACT

The endosperm of *Prunus amygdalus* develops after fertilization into a long narrow tube reaching the chalaza. This tubular formation does not become cellular and functions as a haustorium. Cellularization occurs at the micropilar end of the endosperm and, after the seed has reached its definite size, the cellular endosperm grows at the expense of the nucellus pushing toward the chalaza the haustorial portion.

The membrane of the haustorium is mainly cellulosic and clearly lamellated as shown by electron micrographs of its sections. The X-ray diagrams show lack of orientation through the thickness of the membrane and indicate some slight difference in spacings between the haustorial cellulose and the cellulose of *Cladophora rupestris*.

An interesting feature of the haustorial membrane is the presence of numerous roundish structures, about 4μ in diameter, which show an irregular maltese cross when observed under crossed nicols. The electron micrographs show that these structures correspond to regions of the membrane where the regular lamellation is disturbed, the membrane appearing swollen and the interior of the 'bubble' being occupied by irregular spiral-like coils. In some cases, through stripping of the membrane, some rather thick coiled strands have been isolated.

INTRODUCTION

THE work to be described in this paper originates from the observation of a great number of ring-like structures approximately 4μ in diameter in the haustorial portion of the almond endosperm. These structures are not, so far as I know, described in the existing botanical literature and, because of the presumably complex function of the haustorium, it seemed worth while to investigate them further.

The development of the almond ovule after fertilization can be summarized as follows. During the first period (Text-fig. 1), the whole ovule grows considerably and the embryo-sac develops into a long narrow tube (Pl. 1, Fig. 1), enlarged at both ends, ultimately reaching the chalaza (Pl. 1, Fig. 2) with which it establishes a fairly intimate contact. During the last stage of this phase the upper portion of the endosperm increases in size and begins to become cellular, while the nuclear endosperm remains as a long narrow tube (Pl. 1, Fig. 3). The embryo is still microscopic. Once the nucellus and testa have reached their definite size, the cellular endosperm grows slowly until it occupies all the space left by the disintegrating nucellus; the embryo

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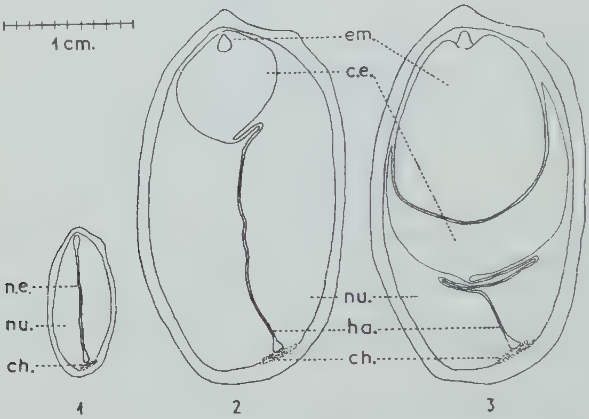
also grows and uses up the endospermic material. The cellular endosperm appears, therefore, as a buffer tissue dividing the lower portion of the nucellus from the growing embryo (Went, 1877; Péchoutre, 1902; Schanderl, 1949; Sarfatti, 1958).



TEXT-FIG. 1. Three stages in the development of the almond seed. (1) *c.* 1·5 month after fertilization: growth of the testa and of the nuclear endosperm; (2) *c.* 3 months after fert.: growth of the cellular endosperm; (3) *c.* 3·5 months after fert.: growth of the embryo.

c. e. = cellular endosperm, *ch.* = chalaza, *ha.* = endosperm haustorium, *em.* = embryo, *n. e.* = nuclear endosperm, *nu.* = nucellus.

The nuclear endosperm, i.e. the haustorial portion, is pushed forward so that at the end of seed maturation it can be found squashed between the cotyledons and the chalazal part of the testa together with the remains of the nucellus and of the cellular endosperm.

It must be borne in mind that the ovular teguments are lined with a continuous inner cuticle, except in the chalazal region (Péchoutre, 1902; Netolitzky, 1926), and that the vascular bundle runs along the raphe and inside the teguments to the region underlying the chalaza (Le Monnier, 1872); there it branches into a number of smaller bundles growing upward towards the micropile, while a great number of conducting elements connect the main branching-point with the chalaza. The nuclear endosperm must function evidently as one long uninterrupted conducting element between the cellular endosperm and the chalaza, and, through the latter, the vascular bundle of placental origin. At the same time it is to be expected that the haustorium also absorbs nutrients from the nucellus until at least this tissue is disorganized; in fact there is a stage when the central part of the seed is nearly empty and all the cell-walls are collapsed, while the lower part of the nucellus is apparently still functioning (Pl. 1, Fig. 2).

When the endosperm is dissected out it is mostly in the central part of the haustorium, which is a tube about 20 mm. long and 0·1 to 0·3 mm. wide, that the roundish structures occur, irregular in shape and varying in size. They are lacking in the lower part of the haustorium where the nucellar cells remain organized for a much longer time. These structures were first detected under the polarizing microscope, but can also be seen in non-polarized light. Between crossed nicols, they stand out as roundish structures much more birefringent than the rest of the membrane, and with a very irregular dark cross (Pl. 2, Fig. 4).

As neither the normal nor the polarizing microscope could give a clear enough picture of these formations it seemed desirable to investigate them with the aid of the electron microscope. The opportunity has also been taken of examining the nature of the crystalline substance in the wall by X-ray diffraction analysis.

MATERIALS AND METHODS

All the almond (*Prunus amygdalus* Stokes) material was collected from trees growing near the Department of Botany, University of Bari (Italy). Some of the material was dissected while fresh, or after treatment with sodium hypochlorite, while some was fixed with various fixing agents (such as formalin, acetic acid-alcohol-formalin, &c.) for sectioning. A number of haustoria were fixed in 2 per cent. osmic acid buffered at approximately pH 6·9.

The material to be examined with the electron microscope was embedded in methacrylates composed of varying proportions of butyl- and methyl-methacrylate. A harder embedding medium, with nearly 1 part methyl- to 3 parts butyl-methacrylate, gave the better results.

For the electron microscopical observations the stripping technique was also used. Small pieces of haustorial membrane were pressed between two adhesive tapes which were then torn apart. This operation can be repeated as many times as necessary and aims at separating the membrane into thin layers which, after shadowing, can be observed in the electron microscope. The material did not strip well, showing a tendency to break up into small fragments, but some useful preparations were obtained. X-ray diagrams were obtained using CuKα radiation collimated to 0·5 mm. diameter and with a flat film about 3 cm. from the specimen. Calibration was achieved as described in the appropriate place below.

The electron microscopy and the X-ray analyses were carried out in the Subdepartment of Plant Biophysics, Botany Department, University of Leeds, during the summer of 1959, but some preliminary observations, which gave a general idea of the problem, were made the previous year by Professor K. Mühlethaler at the Zürich Polytechnic.

RESULTS

(*a*) *Investigations with the optical and polarizing microscope*

The haustorial membrane has a very irregular appearance and its thickness can vary considerably from under 1 to nearly 2μ. It is very wrinkled, at least after dissection, and its outer boundary is often difficult to dissociate from the squashed cell walls of the adjoining empty nucellar cells. It is hardly birefringent on surface view, but its sections show a more appreciable birefringence. It often shows crossed striations, or even seems to be made of interwoven thickish cellulosic strands, but the structure is too variable to allow any clear picture to be presented. The same difficulty applies to the round structures, as regards their distribution, frequency, shape, and general appearance. When they are well evident they look like roundish bubbles rather than rings, quite close to each other and arranged in roughly parallel rows obliquely encircling the haustorium. In polarized light their weak birefringence is sufficient to give marked contrast with the rest of the membrane which remains dark in surface view. They show maltese crosses of the type shown, for instance, in bordered pits of tracheids, indicating therefore a tangential arrangement of the crystallites. The maltese cross is, however, usually very irregular.

(*b*) *Electron microscopical observations*

Electron micrographs of sections show clearly a lamellar structure in the haustorial membrane (Pl. 3, Figs. 6, 7). In most sections, typified by the illustration in Pl. 3, there are regions where the regular run of the lamellae is disturbed and the appearance of these regions is often such as to suggest that they are not mechanical artifacts developed during embedding and sectioning. It appears that these disturbed regions correspond to the bubble-like structures seen in the light microscope. They are normally less than 4μ in diameter, which is to be expected since it would need 100 sections, 200 Å. thick, to cover half the 4μ bubbles and therefore to ensure observation of a median section.

Small fragments of haustorial wall were obtained by stripping thin enough to be observed under the electron microscope. Both in surface view and at various points through the membrane thickness, the lamellae appear to be made mainly of non-oriented microfibrils (Pls. 4, 5), but a tendency can be detected toward orientation in that the microfibrils of one lamella lie roughly at right angles to those of the next lamella. Striations are sometimes present suggesting a cross-structure. In this view the bubble formations appear as irregular spirals found on the stripped fragments, and sometimes as coiled strands which during the stripping may be torn away from the rest of the membrane (Pl. 6).

(*c*) *X-ray diffraction diagrams*

X-ray diffraction diagrams were made of small segments of flattened haustoria pressed together in small stacks, with the X-ray beam both normal and parallel to the surface of the membranes. With the X-ray beam normal to the flattened surfaces no orientation could be detected. With the beam parallel to the surface some orientation is shown (Pl. 2, Fig. 5) because, since the major axes of the microfibrils lie parallel to the surface, the preparation corresponds to a set of coplanar fibrils rotating about an axis perpendicular to this plane. The material shows a low degree of crystallinity but, especially after treatment with 2 per cent. HCl, 4 per cent. KOH, and potassium chlorite, the main arcs were measurable with some accuracy. The arcs corresponding to planes of 2·58 Å. spacings were used for the calibration, but in some cases diagrams of both the haustoria preparations and samples of purified cellulose of *Cladophora rupestris* were recorded on the same plate. The two diagrams appear on alternate quadrants and the arcs can be directly compared.

Diagrams were also made with material treated with 4N KOH. The results are summarized in Table 1.

TABLE 1

Main Spacings in Å. for the Walls of Almond Endosperm Haustoria before and after Treatment with 4N KOH. X-ray Beam Parallel to the Surface

Almond haustoria				
Calibrated on the 2·58 Å spacing	Calibrated on *Cladophora*	*Cladophora* cellulose	Theoretical values	Index
		Before treatment		
2·58 M	2·53	2·58	2·58	040
4·02 E	3·89	3·89	3·93	002
5·53 E	5·43	5·28	5·39	$10\bar{1}$
		After treatment		
			(Mercerized cellulose)	
4·1	—	—	4·42 4·03	$10\bar{1}$ 002
4·28	—	—	4·38	021
7·3	—	—	7·35	101

M = meridional, E = equatorial (see Pl. 2, Fig. 5).

The spacings observed for the material after treatment with 4N KOH show values close to the theoretical ones for mercerized cellulose, particularly the spacing of the 101 planes (7·35 Å.) which differentiates the mercerized from the native cellulose. The spacings of the untreated wall show values which agree fairly well with the theoretical ones for native cellulose, but the correspondence is not exact, particularly when directly compared with *Cladophora* cellulose. The spacing corresponding to the 040 planes is 2·53 Å. for the almond haustorium and 2·58 for *Cladophora*, and the spacings corresponding to the $10\bar{1}$ planes are 5·43 and 5·28 Å. respectively. These differences cannot be attributed to experimental error, such as a difference between the distance of the two samples from the film, since the values differ in opposite senses in these two sets of figures. Moreover it is odd that a reflection corresponding to $10\bar{1}$ planes appears on the equator in these diagrams. Wherever uniplanar orientation has been reported, only the 101 planes are recorded on such a diagram (Preston and Astbury, 1937; Astbury and Preston, 1940; Sisson, 1938; Nicolai and Preston, 1952). The one exception appears to be the case of *Spirogyra* (Kreger, 1957). Uniplanar orientation has not so far been demonstrated in higher plants (Roelofsen, 1959); if this haustorial wall does provide such a demonstration then the orientation is different from that found in most algae.

CONCLUSION

In the absence of data on the genesis and function of the bubble-like structures it is not easy to interpret the morphological data obtained through the optical, polarizing, and electron microscope. The membrane as a whole is fairly thick, heterogeneous in structure and highly wrinkled, but is clearly lamellated and mainly cellulosic. Its chemical nature, already apparent from routine histochemical tests and its birefringence, has been confirmed by the investigation with X-rays. The degree of crystallinity is low and the microfibrils through the whole thickness do not show any high degree of orientation, although the electron micrographs show some tendency toward orientation in each layer with a disposition at right angles in contiguous layers. The X-ray diagrams indicate some slight difference in the spacings between the haustorial cellulose and the cellulose of *Cladophora rupestris* and a uniplanar orientation of the microfibrils different from that prevailing in the algae. The low crystallinity of the material causes the diagrams to be too diffuse to allow a more definite conclusion on this point. Cellulose II is regularly obtained with 4N KOH treatment.

The bubble-like structures can be defined only as regions where the regular lamellation is disturbed, the membrane appearing swollen, and the interior of the 'bubble' being occupied by irregular spiral-like coils: in some cases rather thick coiled strands of microfibrils have been isolated. This is not inconsistent with the figures obtained under the light microscope and with the behaviour of this structure in polarized light.

ACKNOWLEDGEMENTS

The author is greatly indebted to Professor R. D. Preston, F.R.S., for his guidance while working in the Plant Biophysics Subdepartment, Leeds University, and to Professor K. Mühlethaler for the preliminary electron-microscopical observations. He is also grateful to Dr. Eva Frei and Dr. M. C. Probine for help and advice and to Mr. White for technical assistance. This work at Leeds was carried out thanks to a grant from the Consiglio Nazionale delle Ricerche, Rome, Italy.

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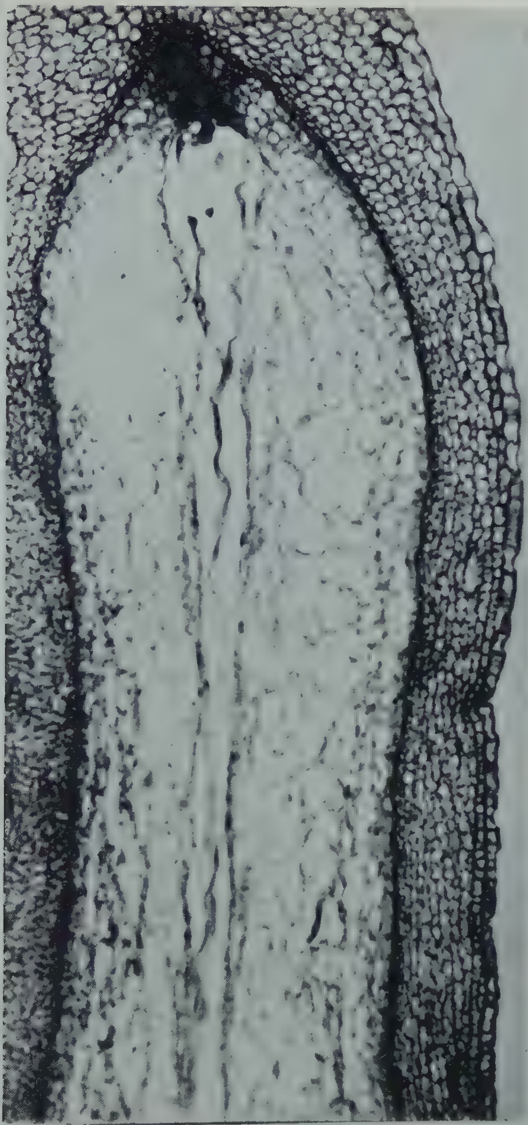

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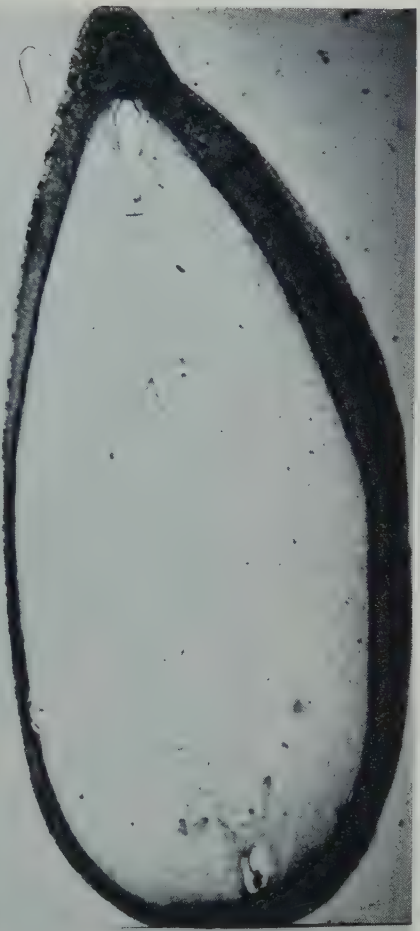

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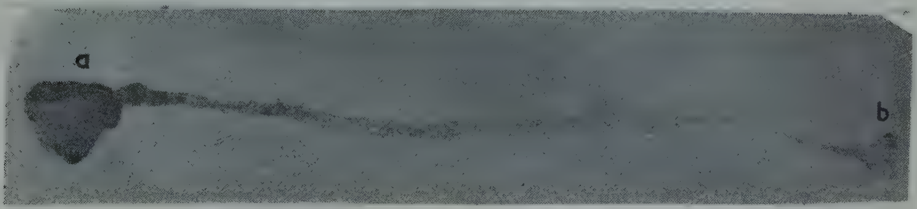

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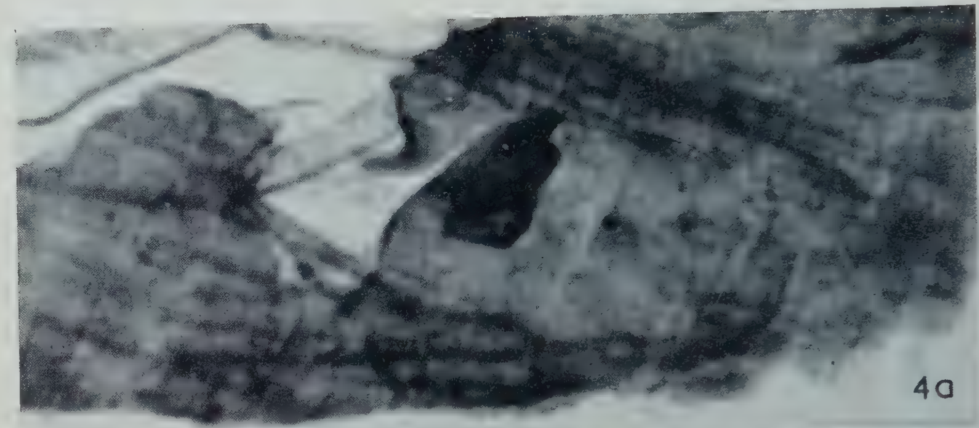

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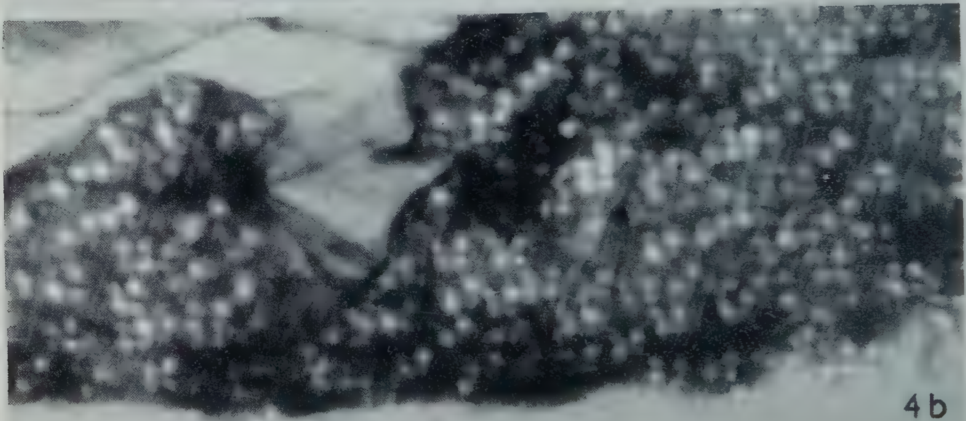

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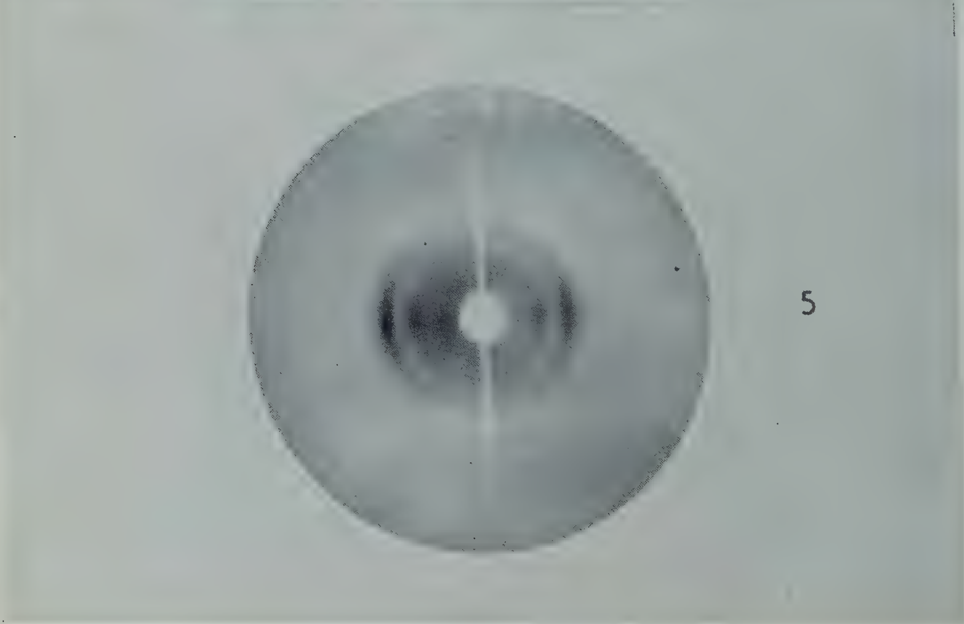

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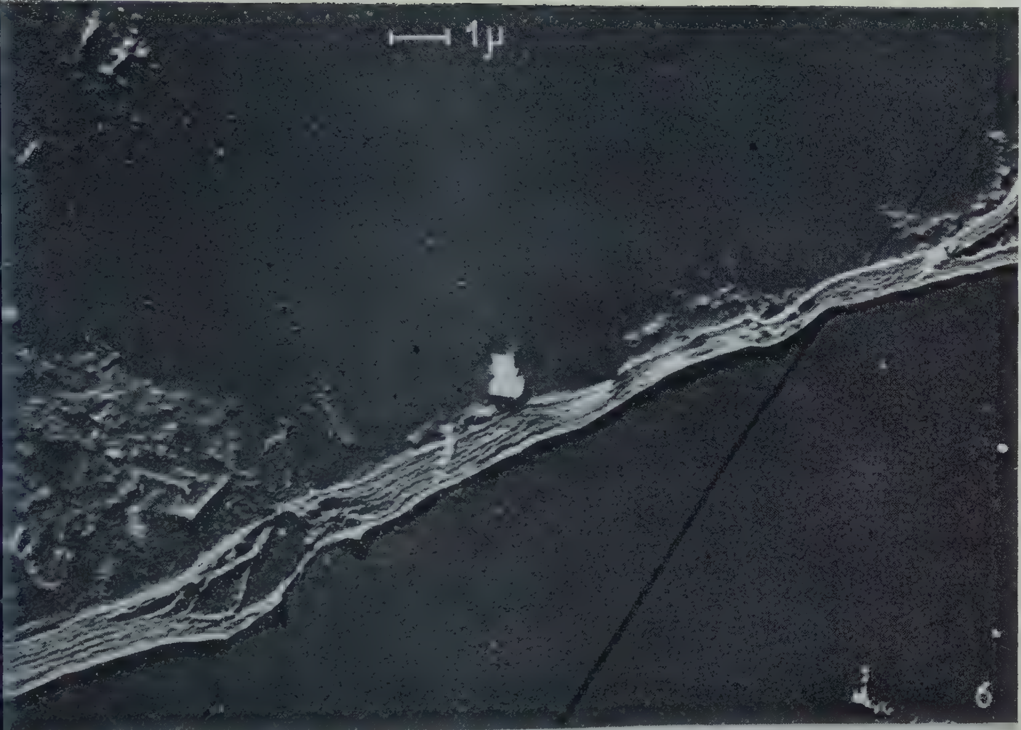

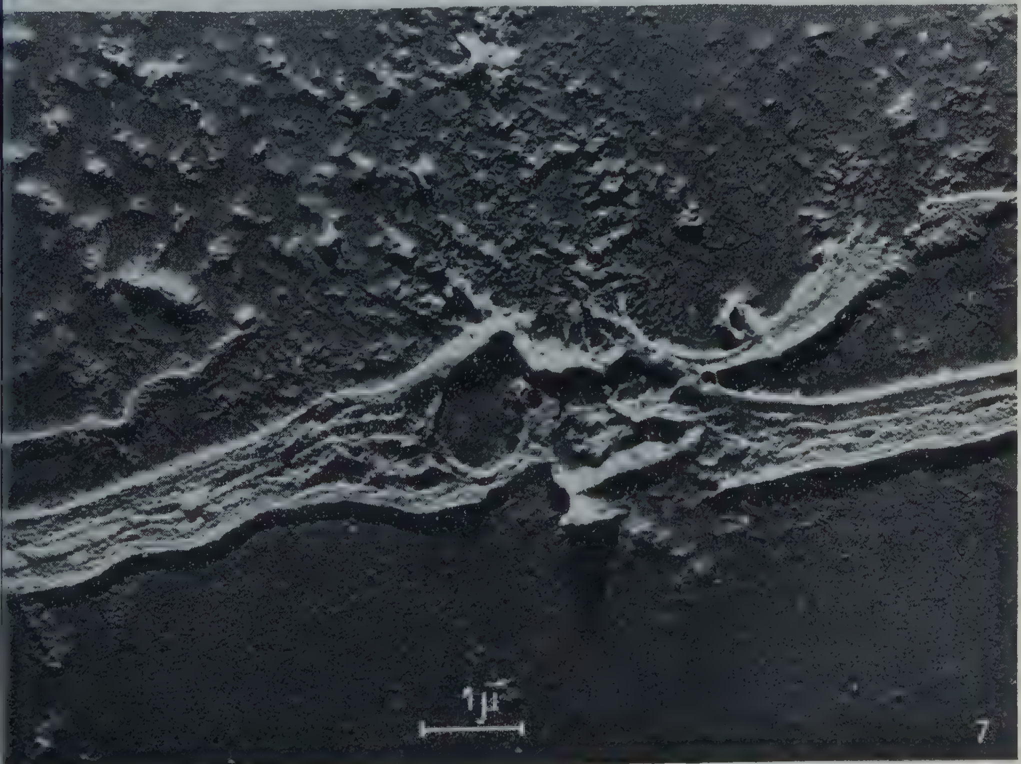

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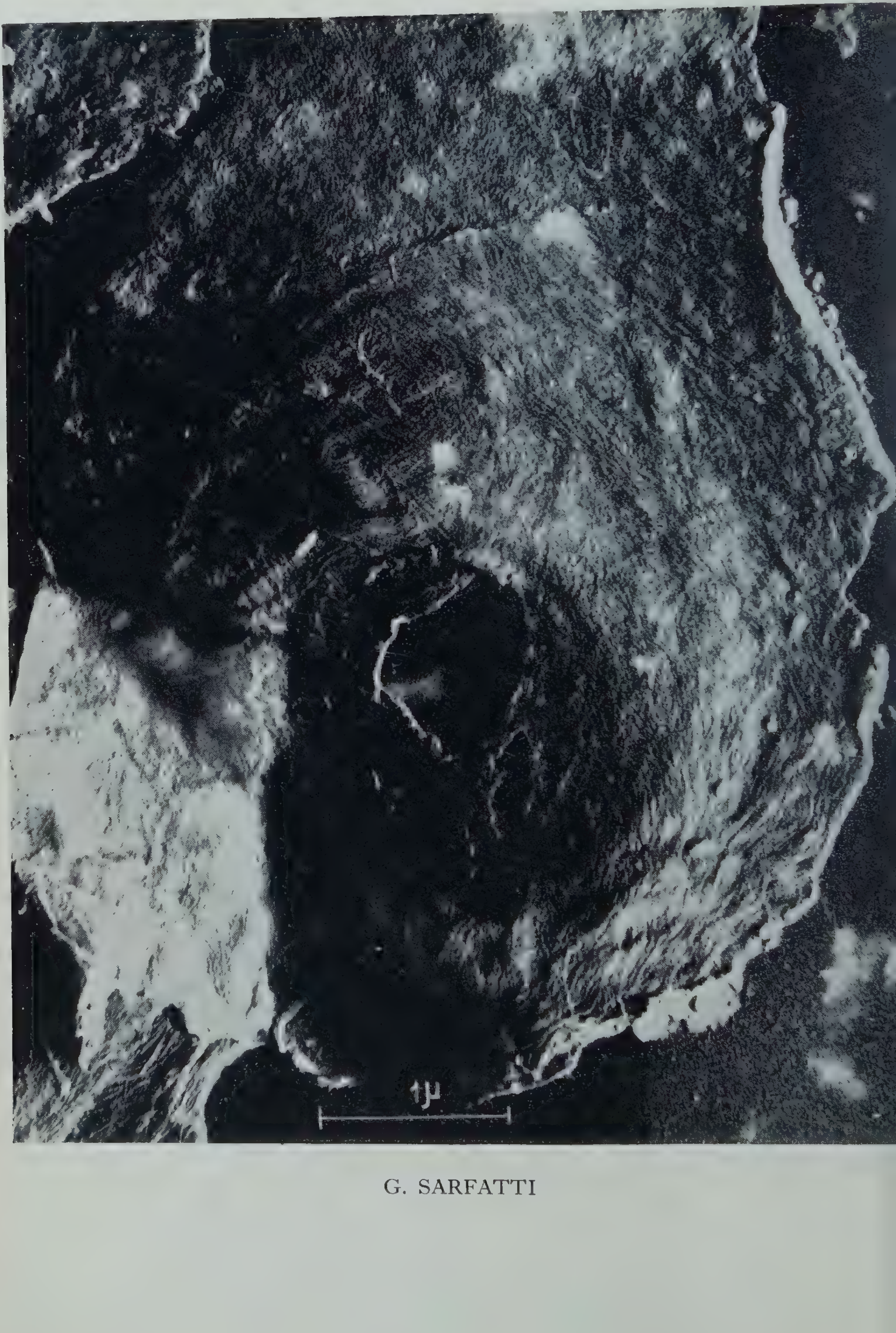

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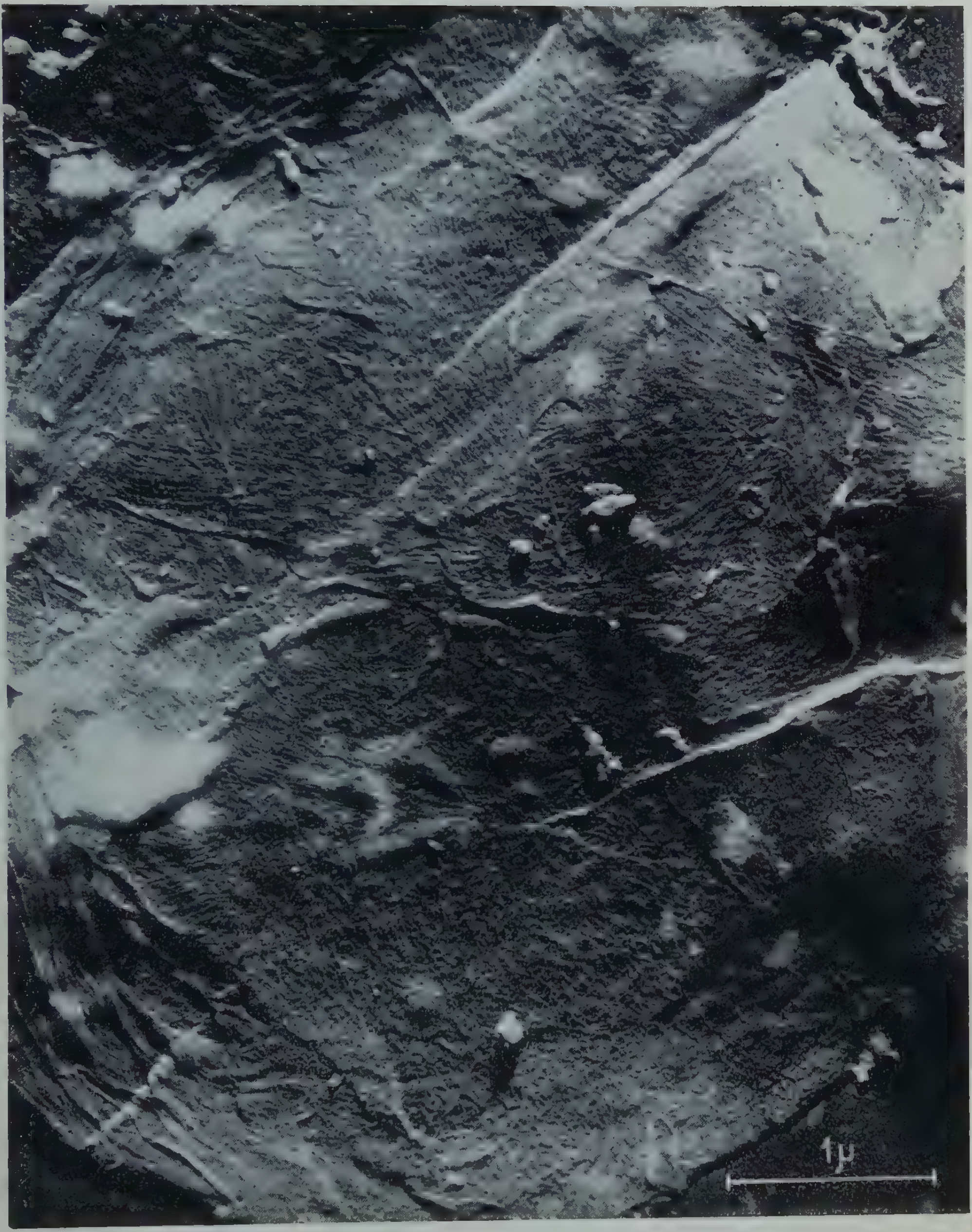

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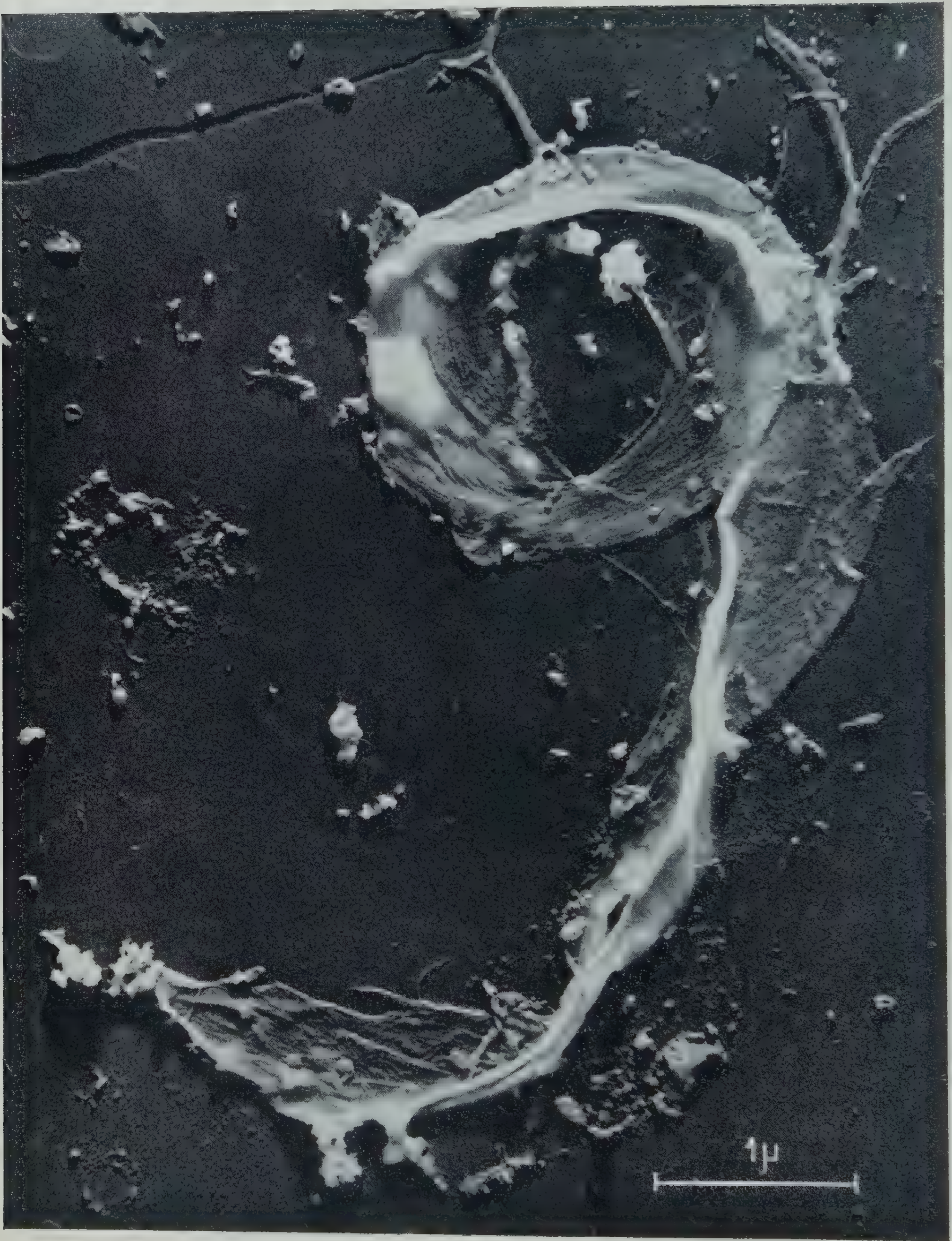

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EXPLANATION OF PLATES 1 TO 6

Plate 1

Fig. 1. Longitudinal section of an almond ovule *c.* 30 days after fertilization, showing growth of nuclear endosperm. (×126)

Fig. 2. Longitudinal section of an almond ovule *c.* 50 days after fertilization. The micropilar and chalazal ends of the haustorium can be seen, together with a small portion towards the centre of the nucellus. (×10)

Fig. 3. A fully developed almond endosperm haustorium with, in (*b*), its chalazal end. In (*a*) the end of the cellular endosperm. (×5)

Plate 2

Fig. 4. A portion of haustorial membrane seen in (A) normal, and (B) polarized light. The round structures, observed in polarized light, show an irregular maltese cross. (×1,000)

Fig. 5. X-ray diagram of almond endosperm haustoria, treated with 2 per cent. HCl, 4 per cent. KOH, and $KClO_2$. Beam parallel to the surface of flattened and stacked material. Cu Kα. Specimen-film distance 2·95 cm. The white line is the shadow of the wire holding an additional beam-stopper introduced between specimen and film to reduce air and collimator scattering.

Plate 3

Fig. 6. Electron micrograph of a section of haustorial membrane. Methacrylate removed, shadowed Pd–Au. Outer surface of membrane uppermost. (×7,200)

Fig. 7. As in Fig. 6. (×13,500)

Plates 4 and 5

Electron micrographs of fragments of stripped haustorial membrane (see text). Pd–Au shadowed. Spiral formation probably part of one of the ring-like structures under investigation. (×27,900)

Plate 6

Electron micrograph as in Plates 4 and 5. A coiled strand torn away from the rest of the membrane.

The Growth of Cacao (*Theobroma cacao*) With and Without Shade

BY

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With one Figure in the Text

ABSTRACT

Evidence is presented that young plants of cacao can tolerate high light intensities if provided with adequate water and nutrient supplies.

PROBABLY the first growth analysis of a tropical tree crop was conducted at the West African Cocoa Research Institute, Ghana, with seedlings of Amelonado cacao (Goodall, 1949, 1950, 1955). The relative growth-rates reported were less than 0·02 g./g./day, which is considerably lower than values which have been found for some other tropical plants (Blackman and Black, 1959); these ranged from the exceptionally high value of 0·33 g./g./day for *Zea mays* to 0·13 g./g./day for *Trifolium subterraneum*. The cacao seedling growth-rate was also much below the range of growth-rates that have been cited for a number of temperate plants (Goodall, 1945) for which values of 0·1 to 0·2 g./g./day are typical. In view of the lack of comparable data for other tropical trees it was not possible to determine whether the low cacao growth-rate was a concomitant of its tree habit of growth. It was also concluded from a study of the effect of illumination on growth-rate that 'cacao seedlings need shade for optimal growth' (Goodall, 1955).

Further growth data have recently been obtained in the course of nutritional experiments with cacao seedlings. It was confirmed that the relative growth-rate of cacao seedlings was low in comparison with other plants, but it was also demonstrated that considerably better growth than that previously reported could be obtained provided suitable precautions were taken to remove several growth-limiting factors. Moreover, the data showed that optimal seedling growth occurred at much higher light intensities than was indicated by previous work (Goodall, 1955). Current views on the role which shade plays in the culture of this important tropical crop are briefly discussed in the light of this observation.

Investigations into methods of conducting nutritional pot experiments with cacao seedlings in the open air at Tafo, Ghana, revealed that shade, inadequate water and nutrient supply, and insect damage were the most important factors limiting growth. Also, observations from field experiments with mature trees indicated that excessive air-movement during dry periods might restrict growth by causing defoliation. It was further considered possible that growth

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could be retarded by the high soil temperatures associated with exposure to full daylight. In the experiments reported here, these factors were varied or controlled as follows: *Shade:* This was varied by adjusting the distance between overhead parallel strips of an opaque material. *Water:* It was known that water relations were unsatisfactory in the type of small basket normally used for raising cacao seedlings in nurseries in Ghana. In these experiments a continuous and adequate water-supply was provided by growing the seedlings in concrete pots which stood in shallow concrete tanks. The water level in these tanks was maintained 8 inches below the soil surface and in contact with the bottom inch of soil in the pots. *Nutrients:* Nitrogen, phosphorus, potassium, and magnesium salts were applied in solution at the rates of 0·3 g. N, 0·25 g. P, 0·2 g. K, and 0·075 g. Mg per plant per month. *Insect control:* Initially, control was attempted by spraying the seedlings fortnightly with a solution containing 2 ounces of gamma-benzenehexachloride per gallon of water. This proved to be satisfactory only under shade. Full control of insect damage to exposed seedlings was eventually obtained by watering the soil, before planting, with one gallon of water containing 4 ounces of *Aldrin*, and subsequently spraying the seedlings twice weekly with a solution containing 2 ounces of *Endrin* per gallon of water. *Air movement:* This was restricted by surrounding the tanks containing the seedlings with slatted screens. *Soil temperature:* A white inert mulch reduced the temperature of exposed soil by approximately 5° C. This had no apparent beneficial effect on germination or subsequent growth.

Some growth measurements taken in the course of three nutritional experiments with seed from open-pollinated flowers of an Amazon variety T73 (Nanay × Iquitos) are compared in Fig. 1 and Table 1 with data previously obtained with Amelonado cacao (Goodall, 1950, 1955). The experiments were as follows. In Expt. 1, seedlings were grown with and without added nutrients under five degrees of shade (Nil, 25%, 50%, 75% and 85% shade), without side protection, sprayed fortnightly with Gamma B.H.C., and provided with an adequate water-supply. In Expt. 2, seedlings were grown with and without added nutrients and an inert mulch, with no shade but with side protection, an adequate water supply, and insect control by using *Aldrin* and *Endrin*. Expt. 3 was of 2^4 NPKMg factorial design, with water-supply, insect control, and side protection as in Expt. 2, but the seedlings were germinated under 85 per cent. shade which was progressively reduced to no shade after 18 weeks, in the manner shown at the foot of Table 1. The design of these nutritional experiments precluded a detailed growth-analysis study, but leaf-area measurements taken at intervals and final plant weights enabled a valid comparison to be made with the earlier work.

The mean relative growth-rate calculated for the seedlings which received the NPKMg treatment in Expt. 3, with the approximate assumptions of an initial 1 g. bean dry weight and an exponential increase in weight, was 0·02 g./g./day. This value supports the earlier conclusion that cacao has a somewhat lower growth-rate than other plants which have been studied (Goodall, 1955). However, in all of these experiments better growth was obtained (Fig. 1, curves 4 to 7) than the best previously reported (Fig. 1, curve 3). The better growth under shade may be partly attributed to the use of the more vigorous Amazon material. This vigour has been clearly shown in field experiments, and in addition 2-fold differences in seedling weights have been demonstrated in the nursery at WACRI (Glendinning, 1960) between some other Amazon varieties and Amelonado. On the other hand, the differences in growth of the exposed seedlings as shown in Table 1, 39-fold in leaf area and 21-fold in dry weight, are much too great to be explained by varietal vigour. These large differences must be the result of improved experimental conditions which have enabled seedlings to be successfully grown in full daylight. Consequently, the general conclusion that 'cacao seedlings need shade for optimal growth' (Goodall, 1955) can no longer be considered correct

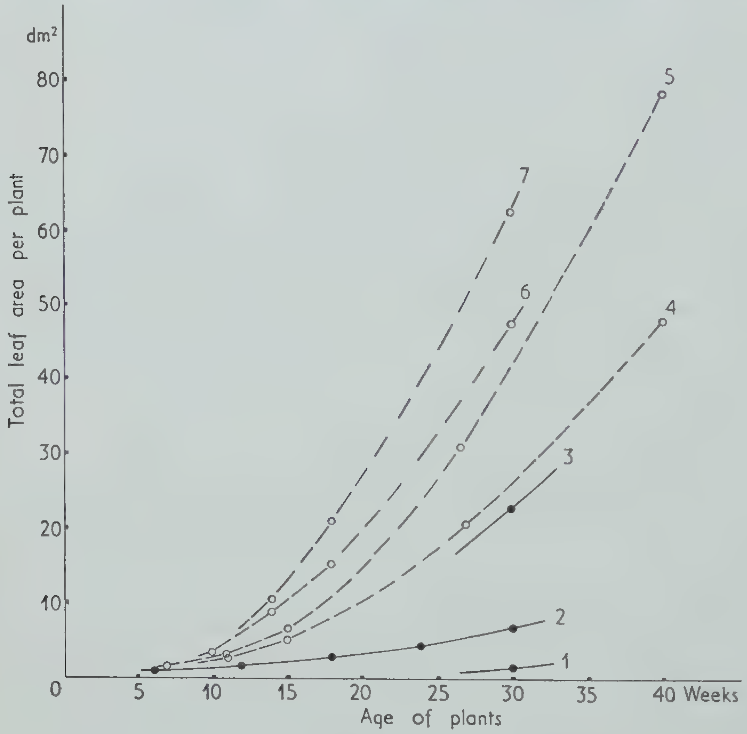

Fig. 1

Key.
1. Goodall, 1955: fully exposed, nutrients not added.
2. Goodall, 1950: heavy shade, nutrients not added.
3. Goodall, 1955: heavy shade, nutrients not added.
4. Experiment 1: heavy shade, nutrients not added.
5. Experiment 1: light shade, nutrients added.
6. Experiment 2: fully exposed, nutrients added.
7. Experiment 3: heavy shade at planting reduced to full exposure after 18 weeks (see Table 1), nutrients added.

Table I

Data from 30-week Old Seedlings Grown under Exposed Conditions

Experiment	Experimental conditions					Growth data at harvest	
	Shade	Water-supply	Nutrients	Pest damage	Side protection	Oven dry weight g.	Total leaf area dm.^{2}
Goodall, 1955	Nil	Intermittent	None added	Possibly severe	Not provided	3·4	1·6
Experiment 2	Nil	Continuous*	None added	Negligible	Provided	27·7	30·5
Experiment 2	Nil	Continuous*	NPK and Mg added	Negligible	Provided	45·8	47·1
Experiment 3	Reduced from heavy to nil†	Continuous*	None added	Negligible	Provided	47·5	44·1
Experiment 3	Reduced from heavy to nil†	Continuous*	NPK and Mg added	Negligible	Provided	71·4	62·1

* See text for method of supplying water continuously.

† Reduced as follows: 0–6 weeks, 85% shade; 6–10 weeks, 75%; 10–14 weeks, 50%; 14–18 weeks, 25%; 18–30 weeks, no shade.

when water and nutrient supply do not limit growth and when insect pests are effectively controlled. The improvement of Expt. 3 over Expt. 2 further suggests that cacao initially needs some shade but that a transition takes place so that within four months, or possibly less, the plants become tolerant of full daylight. The above conclusions have important practical implications.

Although other workers have appreciated that shade is a complex ecological factor, it has previously been assumed that its main function in the culture of cacao was to provide the low light intensities considered necessary for optimal growth. In the light of the experiments noted here and of further evidence from a large field experiment in Ghana (Cunningham and Lamb, 1959), the reduction of light intensity can no longer be regarded as the most useful attribute of the presence of shade. In particular circumstances shade may be chiefly effective, for example, in the following ways: in limiting insect-pest damage; in suppressing weeds and thereby reducing competition for nutrients and water; in maintaining high air humidity by restricting air movement; or in limiting yields by lowering the growth rate of the cacao, thus permitting the use of soils of low nutrient status. The recent experiments suggest that if these ecological factors can be controlled by methods other than the use of shade, for instance by the use of insecticides, weed control, the application of fertilizers, by irrigation or the provision of windbreaks, the highest production of cocoa will be obtained without shade. It is concluded that the interpretation of the role which shade plays in the culture of this important tropical crop must be revised, although it should be emphasized that the actual use of shade need not necessarily be modified in every case.

ACKNOWLEDGEMENTS

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The Relationship between the Critical Leaf Area, Total Chlorophyll Content, and Maximum Growth-rate of some Pasture and Crop Plants

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ABSTRACT

The amounts of leaf and of chlorophyll per unit area of land exposed to light above 400–500 f.c. on sunny days at local noon were determined for pure stands of red clover, white clover, perennial ryegrass, short-rotation ryegrass, maize, kale, cocksfoot, and watercress, growing under near-optimum conditions. The values obtained for the grass species and maize were significantly higher than those of the dicotyledon species.

There was a highly significant correlation ($r = +0{\cdot}912$) between the maximum growth-rates[1] of the different species and the amounts of chlorophyll above the measurement level per unit area of land. However, the index $\frac{\text{Growth-rate}}{\text{Total chlorophyll}}$ was higher for species in which the leaves were disposed horizontally or where flagging occurred (clovers, kale, and maize) than for the grass species, suggesting either that the chlorophyll of the first group of species was more active or efficient in photosynthesis than the chlorophyll in the species with erect leaves, or that a greater proportion of dry matter produced was accumulated in underground organs of these erect-leaved species. Growth-rate data were not available for cocksfoot or watercress.

There was a significant correlation ($r = +0{\cdot}815$) between the leaf-area index absorbing 95 per cent. of the light and the maximum growth-rates of the different species, but this relationship did not take account of the photosynthetic activity of other parts of the plant or of species differences in chlorophyll content.

The results are discussed in relation to the availability and utilization of light energy and carbon dioxide by the species examined.

INTRODUCTION

In an earlier experiment (Brougham, 1956) the rate of growth of pasture following defoliation was shown to reach a maximum when sufficient leaf was produced to intercept 95 per cent. of the incident light at local noon. The term Critical Leaf Area Index was used to describe this amount of leaf and in a subsequent experiment (Brougham, 1958*a*) the Critical L.A.I. was shown to differ markedly for different pasture species. These differences were attributed to differences in habit of growth, and orientation and shape of leaves. Marked seasonal differences in the Critical L.A.I. were also deduced

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[1] In this study, dry-weight increases in plant organs below 1 in. above ground level were not accounted for.

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from measurements made of the amount of light penetrating into the pastures at different elevations of the sun under cloudless conditions.

In view of these findings, it was considered that the amount of chlorophyll per unit area of land exposed to sunlight above the 95 per cent. light-interception level would also differ for pure stands of different species. It was also considered that the maximum growth-rates of pasture and crop species would be related to these amounts of chlorophyll, provided other factors did not limit growth and it could be assumed that chlorophyll activity does not differ between species.

The material presented in this paper was collected to test this hypothesis.

EXPERIMENTAL

The study was carried out at the Grasslands Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand (latitude 40° 30′ S.).

Measurements were made of the areas of leaf (Critical L.A.I.) and the amounts of stalk per unit area of land above the 95 per cent. light-interception level, and the chlorophyll contents of this leaf and stalk were also determined. All these measurements, except two, were made between February 2 and 17, 1960, when the mean elevation of the sun at local noon is 64° and the mean total solar radiation on cloudless days is approximately 680 cal./cm.^{2}/day (maximum noon intensity of 1·25 cal./cm.^{2}/min. or 8,400 f.c.) of horizontal surface, as recorded by an Eppley pyrheliometer.

The species were grown as pure stands under conditions of high soil fertility, and except where stated, irrigation was used to supplement normal rainfall during the months of December and January. They are listed below together with details of sowing dates and methods.

Dicotyledon species:

White clover (*Trifolium repens* L.) broadcast sown September 1959 at the rate of 6 lb. per acre.

Red clover (*Trifolium pratense* L.) broadcast sown October 1958 at the rate of 10 lb. per acre.

Kale (*Brassica oleracea acephala*) broadcast sown December 1959 at the rate of 3 lb. per acre (not irrigated).

Watercress (*Nasturtium officinale* R. Br.) growing in a shallow drain carrying stagnant water 2 in. deep.

Monocotyledon species:

Perennial ryegrass (*Lolium perenne* L.) broadcast sown September 1959 at the rate of 60 lb. per acre.

Short-rotation ryegrass (*Lolium perenne* × *L. multiflorum*) broadcast sown September 1959 at the rate of 50 lb. per acre.

Cocksfoot (*Dactylis glomerata* L.) broadcast sown September 1959 at the rate of 25 lb. per acre.

Maize (*Zea mays* L.) drilled in 7 in. rows December 1959 at the rate of 90 lb. per acre (not irrigated).

The pasture species were sampled when 95 per cent. of the incident light was first intercepted at a level of 1 in. above ground following defoliation, whereas the crop species and watercress were sampled at more advanced stages of growth. White clover, red clover, and short-rotation ryegrass were also sampled at more advanced stages of growth to follow possible changes in the Critical L.A.I. and the amount of chlorophyll above the measurement level, these species being chosen for the following reasons: white clover because previous work (Brougham, 1958*b*) had shown that the Critical L.A.I. varied little with increased growth, short-rotation ryegrass because of its tendency to flag, that is for its leaves to bend towards the horizontal, at advanced stages of growth, and red clover because of its similarities in growth form to subterranean clover which exposes increasing amounts of leaf to light above the 95 per cent. interception level (Black, 1958).

Leaf-area Measurements

The experimental techniques used for determining the 95 per cent. light-interception level and the leaf-area indexes of both the grass and clover species were the same as previously described (Brougham, 1956, 1958*a*). For kale, maize, and watercress, the coring method described for the clover species was also used. In the case of kale and watercress, only the leaf laminae were included in the leaf-area determinations, the remaining petiole tissue being included as stalk. For maize, the chlorophyll-free mid-rib section of each leaf was discarded. At each measurement three separate square-foot samples were taken at random, and leaf-area determinations made for each sample. They were made on the morning following the light measurements.

Chlorophyll Determinations

(1) *Sampling procedure.* Most of the samples for chlorophyll analyses were taken from the field between 9 a.m. and 11 a.m. The exceptions sampled between 4 p.m. and 6 p.m. were those made on February 10 and 11, and the measurements of red clover, short-rotation ryegrass, and perennial ryegrass made on February 17.

Total chlorophyll was determined on duplicate 2 g. samples of both leaf and stalk obtained from the leaf-area separations, or cut directly from the field from herbage similar to that sampled for the leaf-area determinations. For these latter, additional samples were taken for determinations of dry-matter percentage, the average standard errors of these being ±0·45. The standard errors shown in Table 1 were calculated from the determinations of chlorophyll made on the samples obtained directly from the leaf-area separations. These samplings are marked in the table.

A possible source of error could have been introduced by the sampling procedure adopted, as considerable diurnal changes in chlorophyll content have been shown for different species (e.g. Bukatsch, 1940; Bavrina, 1959). However, the results presented in Table 1 show that this effect if present was small. For example, the measurements for red clover and short-rotation ryegrass on February 17 were made on samples taken in the afternoon, while the measurements made on the other dates were for samples taken in the morning. Little variation in chlorophyll content is shown, although this could have been due to the fact that chlorophyll content was determined on random samplings of leaves receiving levels of light from 400 to 8,000 f.c. Over such a range of light levels a number of interactions between chlorophyll content of leaves, light-energy levels, and time of exposure to these levels would occur, as is instanced by the work of Bohning (1949).

(2) *Chlorophyll determinations.* The analyses were made immediately following sampling. After grinding the green material with pestle and mortar, the chlorophyll in each sample was extracted with a mixture of acetone and ethyl alcohol (4:1 by volume) by repeated homogenizing in a blender. The macerate was then made up to 100 ml. with the acetone-alcohol mixture, and after thorough stirring an aliquot of the crude extract was centrifuged for 5 min. at approximately 3,000 r.p.m. The supernatant (chlorophyll solution) was then decanted from the residue (colourless cell and cell-wall constituents), 1 ml. diluted 10-fold, and the amount of chlorophyll *a* and *b* determined by measuring the optical density on a Beckmann spectrophotometer at 663 and 645 mμ (Arnon, 1949). The check reading at 652 mμ was also carried out. The average standard errors of the determinations for duplicate 2 g. leaf samples obtained from separate leaf-area samples was $\pm 0{\cdot}25$ mg.

(3) *Growth-rate determinations.* The growth-rate data were obtained from a number of experiments carried out at the Grasslands Division, D.S.I.R., and Massey Agricultural College during the past 5 years. Some of these data are as yet unpublished. Growth-rate was determined as in previous experiments (Brougham, 1956, 1959), although the intervals between measurements differed, ranging from one day for perennial ryegrass to 2–4 weeks for the crop species. Maximum growth-rates were determined by fitting regressions, of as high as third order where necessary, to the raw data. Each growth-rate value shown is for growth after the Critical L.A.I. was reached, but before factors limiting to growth became operative. They represent maximum growth-rates at a time of the year (December–February) when the amount of light energy is at a maximum for this locality and temperatures are within the limits found by Mitchell (1955, 1956) to be favourable for the growth of the grass and clover species. No data were available for the growth of cocksfoot or watercress.

RESULTS

Critical L.A.I. The Critical L.A.I. values shown in Table 1 for the ryegrasses and white clover are very similar to those previously obtained (Brougham, 1958*a*) and the value for kale lies within the range 3–4, calculated by Watson (1958) as that necessary for maximum growth-rate. For the other species, those with horizontally disposed leaves (red clover and watercress) exposed significantly less leaf to light above an intensity of 400–500 f.c.

TABLE I

Critical L.A.I., Percentage Light Interception, and Chlorophyll Content of the Different Species at Each Sampling

Species	Date of sampling (Feb.)	Days of growth from defoliation or sowing	Height of stand and sampling level (cm.)	% Light interception	Critical L.A.I.	Chlorophyll content (mg.)					
						Per g. of green weight	Per g. of dry weight	Per Critical L.A.I. (sq. ft.)			Per sq. ft. of herbage
								Leaf	Stalk	Total	
White clover	3*	24	14 (3)	94	2·7±0·2	2·8	14·3	115	5	120	44
	10*	31	20 (12)	96	3·1 ,,	2·8	15·9	124	7	131	42
	17	38	23 (15)	95	3·2 ,,	2·8	15·1	128	9	137	43
Red clover	2*	22	17 (7)	94	3·0±0·4	3·0	16·5	122	14	136	45
	9*	29	24 (13)	95	3·8 ,,	2·7	14·7	152	27	179	47
	17	37	38 (24)	96	4·6 ,,	3·0	13·8	171	21	192	42
	28	48	52 (34)	95	5·0 ,,	2·9	14·1	184	22	206	45
Kale	2*	54	55 (25)	96	3·1±0·2	1·5	10·9	128	5	133	43
Watercress	2*	—	45 (15)	97	5·2±0·2	1·4	13·6	137	29	166	32
Short-rotation ryegrass	7*	24	20 (3)	95	6·8±0·5	2·0	11·8	233	—	233	35
	17	34	30 (7)	95	6·1 ,,	2·4	12·6	226	—	226	37
Perennial ryegrass	17	26	25 (3)	95	6·0±0·6	1·9	9·9	209	—	209	35
Cocksfoot	11	28	20 (3)	95	6·4±0·5	2·4	11·6	234	—	234	37
Maize	3*	55	105 (55)	96	8·6±0·6	1·9	13·1	263	3	266	31
	21	73	200 (165)	95	6·1 ,,	3·1	15·8	297	—	297	49
Average S.E.						±0·13	—	±19·1	±1·9	±19·2	±3·0

* The average S.E.s shown were obtained from analyses of these data only.

(i.e. 5 per cent. of the incident light) than the species with more erect leaves (ryegrasses, cocksfoot, and maize).

Of the four species sampled twice or more, significant changes in the Critical L.A.I. with increased growth were recorded for red clover and maize. The increases shown for red clover are attributed to stem elongation, while the decrease recorded for maize is attributed to flagging of leaves. Only small differences were obtained for white clover because of its uniform habit of growth once most of the light is intercepted by leaves (Brougham, 1958*b*), and although some flagging occurred in the stand of short-rotation ryegrass, the difference recorded was not significant.

The Critical L.A.I. of watercress was significantly higher than the values obtained for the other dicotyledon species except red clover at later stages of growth. This high value can be accounted for by the lower measurement level (97 per cent. light interception) and a probable higher transmission of light through the leaves because of their high water content (cf. Rabinowitch, chap. 22, 1945).

Chlorophyll content. The results presented in Table 1 showing chlorophyll content expressed as mg. per g. green weight, dry weight, and unit area of herbage, in general conform with results obtained by a number of workers for a large number of species (cf. Rabinowitch, chap. 15, 1945). As a percentage of green weight, the mean value for all samplings (0·24 per cent.) is very similar to the mean obtained for 600 species investigated by Lubimenko (1928), and as percentage of dry weight, the values obtained are within the range normally found. Comparisons between species show that the chlorophyll contents of the clover species and maize at the second sampling were significantly higher than those of the other species examined. These differences can be explained in terms of differences in leaf thickness and composition, water content of leaves, and chloroplast number per unit area of leaf.

When the chlorophyll content is related to the amount of leaf and stalk per unit area of land, receiving light above an intensity of 400–500 f.c., some marked differences between species are shown. The total chlorophyll value for maize at the last sampling was significantly higher than all others, and except for red clover at the last sampling, the values for the dicotyledon species were significantly lower than those of the monocotyledon species.

Also shown in Table 1 are the amounts of chlorophyll per unit area of herbage for the different species. The mean value of all measurements, when converted to chlorophyll content per 100 cm.^{2} of leaf, is 4·4 mg., a value very similar to that obtained by Seybold and Egle (1940) for a number of species. Comparisons between species show that, except for watercress, the chlorophyll contents per unit area of herbage of the dicotyledon species were significantly higher than those of the monocotyledon species, except for maize at the second sampling.

Growth-rate. The results presented in Table 2 show marked differences in the estimated maximum daily growth of the different species. From these data, the maximum growth-rate of maize in this locality is approximately

TABLE 2

Details of Experiments and Estimates of Maximum Growth-rates

Species	Height of defoliation (cm.)	Dates of measurement		Number of measurements	Replications	Maximum growth-rate*	Source of data
		Initial	Final				
White clover	3	Dec. 4, 1956	Jan. 5, 1957	8	4	121 ± 7	Brougham (1958*a*)
Red clover	10	Dec. 5, 1955	Dec. 23, 1955	6	9	170 ± 7	Brougham *et al.* (1958)
Red clover	23	Dec. 5, 1955	Dec. 23, 1955	6	9	207 ± 13	,, ,,
Kale	—	Dec. 15, 1955	Feb. 15, 1956	3	8	140 ± 12	Sears (unpublished)
Kale	—	Jan. 11, 1957	Mar. 22, 1957	8	12	115 ± 7	Frame (1957)
Short-rotation ryegrass	3	Dec. 4, 1956	Jan. 5, 1957	8	4	169 ± 9	Brougham (1958*a*)
Perennial ryegrass	3	Dec. 12, 1959	Dec. 19, 1959	8	4	156 ± 7	Brougham (unpublished)
Maize	—	Jan. 14, 1956	Mar. 13, 1956	3	9	261 ± 18	Sears (unpublished)

* Pounds of dry matter per acre per day (*c.* kg./Ha.).

twice that of white clover or kale. It also produces significantly greater amounts of dry matter per day than any of the other clover and grass species examined.

In an attempt to explain these differences, correlation coefficients were calculated relating maximum daily growth-rates to Critical L.A.I. or total chlorophyll contents of herbage above the 95 per cent. light-interception level

TABLE 3

Growth-rates per Unit of Leaf Area and Chlorophyll and Correlation Coefficients

Species	Maximum growth-rate*	Mean Critical L.A.I.	Total chlorophyll (mg.) per sq. ft.† of herbage	Growth-rate	
				Critical L.A.I.	Total chlorophyll
White clover . .	121±7	3·00±0·2	129±19	40·3	0·936
Red clover . .	188±7	4·80±0·4	199 ,,	39·3	0·947
Kale . . .	127±7	3·10±0·2	133 ,,	41·1	0·958
Short-rotation ryegrass	169±9	6·45±0·5	229 ,,	26·1	0·735
Perennial ryegrass .	156±7	6·00±0·6	209 ,,	25·9	0·744
Maize . . .	261±18	7·35±0·6	281 ,,	35·6	0·928
Correlation coefficient				+0·815 $p = 0{\cdot}05$	+0·912 $p = 0{\cdot}01$

* Pounds of dry matter per acre per day (≡ *c.* kg./Ha./day).

† *c.* 0·1 sq. m.

for all species. These are shown in Table 3. The figures are the means for all sampling dates shown in Tables 1 and 2 for all species except red clover. For this species, only the data for the last two samplings have been used because of the marked changes in Critical L.A.I. and total chlorophyll content with stage of growth. These means were considered to be more realistic estimates, corresponding to the stage of growth at which maximum daily growth-rate occurred.

The correlation coefficients indicate that the maximum daily growth-rates of the species are significantly related to both the Critical L.A.I. ($r = +0{\cdot}815$) and the amount of chlorophyll per unit area of land above the 95 per cent. light-interception level ($r = +0{\cdot}912$).

DISCUSSION

Although optimum requirements vary for different species, in this location, at the time of the year when all measurements were made, and under the conditions in which the species were growing, optimum environmental requirements would be closely approached. The relationships obtained therefore apply to near-maximum growth in this and similar localities.

The data presented in Table 3 show that the maximum growth-rate of pure stands of the species studied is closely related to the amount of chlorophyll in the leaf and stalk above the 95 per cent. light-interception level. Thus the total amount of chlorophyll per unit area of land exposed to light above an intensity of 400–500 f.c., and the amount of CO_2 available for utilization by chlorophyll, would be the limiting factors to increased growth.

The results presented in Table 3 also suggest that the activity of chlorophyll of the species studied is similar, this being particularly so for the three dicotyledon species and maize (the index $\frac{\text{Growth-rate}}{\text{Total chlorophyll}}$ was markedly similar for these species). This suggests that the concentration of CO_2 available for growth and the utilization of it by the chloroplasts was the same for these species. It is also considered that growth of the clover species, kale, and maize was not seriously limited by the supply of CO_2. Differential respiration during the hours of darkness could account for the differences recorded (double) in the maximum daily growth-rate of these species, but as suggested by Watson (1956), such marked differences in rates of respiration seem unlikely.

Although no measurements of CO_2 concentration were made, it was noticeable during the leaf-area samplings of the clover and kale stands that the highest yellowed leaves were found slightly below the 95 per cent. light-interception level. From this, and previous work (Brougham, 1958*a*), in which it was shown that leaf senescence, and hence CO_2 release, in white clover stands occurred very rapidly under intense shading, it is probable that the amount of CO_2 available for growth would at least be as high as atmospheric concentrations. This has been shown for grass stands by Fuller (1948). For maize, Verduin and Loomis (1944) found CO_2 values of 0·455 mg. per litre of air, 100 cm. above ground level in the late morning hours, but even under such conditions, these authors considered that there was no evidence of a CO_2 limit to growth. It is significant, however, that the intensity of light at ground level in this crop was 30 to 40 per cent. of daylight. Because of the openness of this stand, CO_2 requirements would be lower than in stands where a higher percentage of light is intercepted by leaf.

The index $\frac{\text{Growth-rate}}{\text{Total chlorophyll}}$ was lower for the ryegrasses than for the other species examined (Table 3). Although growth-rate data were not available for cocksfoot, from an examination of Table 1, and of dry-matter yields of this species relative to the ryegrasses from other data, it is considered that the index for cocksfoot would also be lower. In determining the growth-rates of the different species, no account was taken of dry-matter increases in plant organs below a measurement level of 1 in. from the ground. The lower indexes shown in Table 3 for the grass species could, therefore, have been due to a higher proportion of the dry matter produced being accumulated in the underground organs of these species. However, the result is similar to that obtained by Watson (1947), who showed that the net assimilation rates (N.A.R.) of dicotyledonous root crops were higher than those of cereal crops, using dry-matter increases of both above and below ground plant organs in the calculations of N.A.R. In a subsequent study (Watson, 1958), the N.A.R. of different species were shown to be dependent on the amounts of leaf present.

A number of other factors could be used to explain the differences in the index $\frac{\text{Growth-rate}}{\text{Total chlorophyll}}$ between the grass species and the other species examined, and for the similarity of the index for the species in each group. Differences in the amount of CO_2 available for growth between the grass stands and those of the other group of species, because of differences in the life span of leaves, could account for the differences recorded, while species differences in the amount of chlorophyll above the 95 per cent. light-interception level because of diurnal changes in amounts of leaf pigments present would influence CO_2 utilization. The phenomenon of 'Mid-day Depression' (Thomas and Hill, 1949), stomata closure, and differences in leaf structure between species, particularly in stomata number and size per unit area of leaf and in the amount of free space between cells, would also affect movement of CO_2 to the chloroplasts, while chloroplast movement during the day in leaves of different shape, orientated at different angles to the sun, would probably influence the amount of light energy available at the chloroplast site for photosynthesis. Differences in the utilization of this energy throughout the day under conditions of diffuse or low light intensities (Kasanaga and Monsi, 1954) would also affect the relationships obtained for the different species.

The correlation obtained between the Critical L.A.I. and maximum daily growth-rate was also significant, although this relationship was not as close as that already discussed. The chlorophyll content of herbage above the 95 per cent. light-interception level accounted for the photosynthetic activity of the chloroplasts in the leaf, stalk, petiole, and sheath tissue of the different species, whereas the Critical L.A.I. is a measure of the photosynthetic activity of the leaf only. Depending on the habit and stage of growth of different species, the photosynthetic activity of other parts of the plant can be appreciable (Watson and Norman, 1939; Porter *et al.*, 1950; Thorne, 1959). The index $\frac{\text{Growth-rate}}{\text{Total chlorophyll}}$ also takes account of differences in chlorophyll content per unit area of herbage. Thus a closer correlation between the total chlorophyll of the leaf and stalk above the 95 per cent. light-interception level and maximum daily growth would be expected, provided the concentration of CO_2 was non-limiting or was utilized uniformly by the chlorophyll of the different species.

The results presented above show that the production of dry matter by pasture and crop species is ultimately limited by the amount of chlorophyll per unit area of land exposed to light levels above compensation point. This is influenced by the light-intercepting capacity of the leaves of each species, which is in turn determined by the shape and orientation of the leaves to the angle of incidence of light, and the transmission of light through the leaves. The results also suggest, either that the chlorophyll in species with horizontally disposed or flagged leaves is more active or efficient in converting CO_2 than that in species with more erect leaves, or that a greater proportion of the dry matter produced is accumulated in underground organs of the erect-leaved species. These results are of importance in selecting plant material for increased productivity.

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ADDENDUM

Since submitting this article for publication results published by Bray (1960) have shown a highly significant correlation between the dry weights of the above-ground crop of several annual herbaceous stands and the concentration of chlorophyll per unit area of land surface. The above-ground dry weights represented approximate annual net increments of the aerial plant portions, whereas chlorophyll content was determined for aliquot samples of herbage of all above-ground plant material excluding duff and litter. Thus the relationship shown was for the stands at their maximum total yield. The results are complementary to those obtained in this study, those of Bray demonstrating the feasibility of using total chlorophyll content as an index of net annual productivity of undisturbed herbaceous stands, and those presented in this paper showing the possibilities of using total chlorophyll content of herbage above the 95 per cent. light-interception level as an index of maximum growth-rate for pasture and crop plants.

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Bray, J. R., 1960: The Chlorophyll Content of Some Native and Managed Plant Communities in Central Minnesota. Canad. J. Bot., **38,** 313–33.

The Influence of certain simple Nitrogenous Compounds on Growth and Sporulation of *Chaetomium globosum*

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ABSTRACT

Chaetomium globosum grows vigorously on media containing, as source of nitrogen, ammonium pyruvate, lactate, succinate or tartrate, or urea. Most common amino-acids, except lysine, permit growth to varying degrees. The carbon of those compounds which sustain growth is also utilized by *C. globosum*, but usually only when glucose is also available. Sporulation is vigorous on media containing ammonium pyruvate or lactate, α-alanine or aspartic acid; less so on other amino-acids, and very weak on ammonium tartrate. Vigour of fruiting was apparently connected with the presence of organic phosphates in the media, and it is suggested that the favourable influence of pyruvate, lactate, and α-alanine is due to their ready transformation into 3-phosphoglyceric acid; this transformation has been demonstrated for α-alanine by the use of the ^{14}C-labelled compound.

INTRODUCTION

SOME investigations into the metabolism of *Chaetomium globosum*, with particular reference to the production of perithecia, have been reported in a series of papers by Buston and co-workers (Buston and Basu, 1948; Buston and King, 1951; Buston, Jabbar, and Etheridge, 1953; Buston and Khan, 1956). The mineral salts–sugar medium used by these workers contained sodium nitrate as a source of nitrogen, but preliminary experiments (Basu, 1948; Jabbar, 1954) showed that growth and sporulation were notably affected when this salt was supplemented or replaced by other nitrogenous compounds. The experiments now described were undertaken to study in more detail the effect of a variety of simple nitrogenous compounds on the growth and sporulation of this organism.

MATERIALS AND METHODS

The strain of *C. globosum* used was that originally isolated by Basu from a piece of brown paper rotting in contact with moist soil in India, and designated by him No. 79. It has since been maintained in stock culture on cellulose agar slopes. The general culture methods have been described by Buston and King (1951). In all cultures the substance used as a source of nitrogen was added to the medium to give a final concentration equivalent to 0·033 per cent. N. When an organic compound of nitrogen was used, the concentration of glucose in the medium (normally 0·5 per cent.) was reduced by an amount equivalent to the carbon present in the nitrogenous compound, unless otherwise stated.

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Growth in liquid media was measured as the average dry weight of mycelium harvested from a suitable number of cultures (4–6); on agar media, growth was estimated from colony diameter (the mean of 2 measurements per plate, taken at right angles). The amount of sporulation was estimated by a count of 'perithecial frequency' as described by Asthana and Hawker (1936). Perithecia which had begun to develop hairs were counted even though these were not fully pigmented and contained no mature spores; less developed perithecia (initials) were not counted.

RESULTS

In a general survey, the nitrogenous compounds tested included inorganic nitrate, ammonium salts, amides, and a wide range of amino-acids. The influence of these compounds on the mycelial growth of *C. globosum* is summarized in Table 1, and in Table 2 the effect of some of them on sporulation on solid media is recorded.

The results given in Table 1 show that differences exist not only in the maximum amounts of mycelial growth promoted by different nitrogenous compounds, but also in the ease with which the compounds are assimilated, as shown by the rate at which growth proceeds. Differences in the vigour of sporulation on different media were very evident, and bore little relation to the amount of vegetative growth. Thus the ammonium salts of pyruvic, lactic, succinic, and tartaric acids gave almost the same maximum vegetative growth (although in liquid media growth on lactate was very much slower), but the amount of fruiting on tartrate was very much less than on the other media. Ammonium lactate and pyruvate, α-alanine, aspartic acid, and asparagine were outstanding in encouraging fruiting on agar media. The close structural relationship between the first three of these suggested that, whatever the mechanism by which these compounds stimulated sporulation, it was shared by all three; and it seemed reasonable to assume that the stimulus came from the carbon skeleton of the molecule rather than from the nitrogen-containing radical. Previous work has shown that sporulation in some fungi is enhanced by the presence of small amounts of hexose phosphates or other phosphorylated intermediates of glycolysis in the medium at some phase during the life of the organism (Hawker, 1947, 1948), and the marked activity of pyruvate, lactate, and α-alanine in stimulating fruiting of *C. globosum* suggested that their effect might be due to a direct and relatively easy transformation of all these compounds into 3-phosphoglyceric acid. In order to test this possibility, portions of liquid media in which the fungus was growing were examined chromatographically (about the time when vigorous fruiting was observed) for the presence of organic phosphates (Bandurski and Axelrod, 1951). Glucose-6-phosphate, fructose-1,6-diphosphate, and 3-phosphoglyceric acid were detected in media based on pyruvate, lactate, and α-alanine, although the amounts of the different phosphates varied widely. On the other hand, similar experiments in which the source of nitrogen was ammonium tartrate (which, as seen above, does not encourage sporulation) revealed only the

TABLE 1

Effect of Nitrogenous Compounds on Growth of C. globosum *in Liquid Media*

Source of nitrogen	Initial glucose concn. (g./l.)	Mycelial growth (mg. dry wt., average of 4 cultures) Early (6–8 days)	Maximum
$NaNO_3$	5·0	17	30
Amm. phosphate	5·0	13	33
Urea	4·65	18	35
$NaNO_3$	3·6	..	24
Amm. succinate	3·6	25	32
Amm. tartrate	3·6	27	31
Glycine	3·6	6	22*
Asparagin	3·6	18	32
$NaNO_3$	2·9	..	17
Amm. pyruvate	2·9	21	32
Amm. lactate	2·9	10	29
α-alanine	2·9	16	26
Serine	2·9	..	23
Lysine	2·9	(<1)	(3)
$NaNO_3$	2·2	..	13·5
Aspartic acid	2·2	14	24
$NaNO_3$	1·5	..	10
Valine	1·5	17 mg. on day 20	
Glutamic acid	1·5	12	26
Leucine	0·8	12 mg. on day 21	
Phenylalanine	0	0	0
Phenylalanine	3·6	4	10†

* Medium strongly pigmented. † Cf. alanine.

All amino-acids were L-isomers; initial pH 5·5 throughout.

(Most of the figures recorded for 'maximum growth' were obtained in independent experiments by Miss Judith Lee, to whom the authors' thanks are due.)

TABLE 2

Effect of Nitrogenous Compounds on Growth and Perithecial Frequency (P.F.) in 6-day Cultures of C. globosum *(agar media)*

Source of nitrogen	Mean diameter of colony (mm.)	P.F. (total)	P.F. (large perithecia)
$NaNO_3$	29·5	8·2	0·9
Glycine	34	15	1
α-alanine	39	24	4·7
Serine	42*	4·9	0
Aspartic acid	30	23	7·5
Asparagin	31	21	1·5
Glutamic acid	49*	11·8	1·8
Amm. pyruvate	32	24	4
Amm. lactate	28	25	7
Amm. succinate	30	8	1
Amm. tartrate	40	5	0·3

* Growth thinner than on other media.

presence of 3-phosphoglyceric acid in small traces. Quantitative estimation of individual phosphates was not attempted, but the chromatograms indicated that the phosphate formed in the greatest quantity was 3-phosphoglyceric acid (in the alanine medium). Estimations of the total organic phosphate in some of the media were made, and the results are given in Table 3.

Pyruvic acid (a probable intermediate between alanine and 3-phosphoglyceric acid) was detected in the alanine medium on the 8th day, and was also readily produced on incubating an acetone-dried powder of *C. globosum* mycelium in 0·1 *M* α-alanine, pH 6·0, at 30°. These observations were taken to indicate a direct transformation of α-alanine to pyruvic acid and thence to 3-phosphoglyceric acid. However, since all media contained also glucose which would itself give rise to the same phosphates, direct proof of the conversion of the intact 3-carbon skeleton of α-alanine, pyruvic acid and lactic acid into 3-phosphoglyceric acid was lacking. Some attempts were therefore made to grow *C. globosum* on media in which the nitrogenous compound served also as the sole source of carbon. Among a variety of substances tested, only α-alanine and glutamic acid permitted growth in the absence of glucose; with these growth was slow and scanty. Since these experiments were inconclusive, evidence was sought by using ^{14}C-labelled α-alanine, of which 1·4 mg. (total activity 1 mC.) was incorporated in 1,200 ml. of the medium containing α-alanine. At suitable intervals during the period of incubation, pairs of flasks were taken, the mycelia filtered off, and the filtrates were examined chromatographically for simple saccharides (Partridge, 1948; Buchan and Savage, 1952), amino-acids (Datta, Dent, and Harris, 1950), and organic phosphates (Bandurski and Axelrod, 1951). Radioactivity on the chromatograms was detected by autoradiography, and its intensity was estimated visually. Uptake of radioactive carbon by the mycelium was followed by testing standard amounts of the dry tissue with an end-window Geiger counter.

TABLE 3

Total Organic Phosphate in Liquid Culture Media, 8th day

Source of nitrogen	Organic P, μg./ml.	Relative amount of fruiting
$NaNO_3$	2·3	2
α-alanine	4·1	4
Aspartic acid . . .	4·1	4
Serine	2·2	3
Amm. tartrate . . .	1·1	< 1

Results of analyses of samples of the filtered media taken during the period 120 hr. to 184 hr. after inoculation are summarized in Table 4. Attention may be drawn to the wide variety of organic phosphates that were detected during the period; most of these appeared transiently and had disappeared or greatly diminished by the 184th hr. Those present in the greatest amount were fructose-1,6-diphosphate and, especially, 3-phosphoglyceric acid. In no instance did any phosphate in the early samples (up to *c.* 136 hr.) show any radioactivity, but activity appeared rather suddenly in all at some time between 142 and 148 hr., and was most marked in fructose diphosphate and phosphoglyceric acid, in which it reached a maximum at 160 hr. Simple saccharides (in addition to the glucose of the medium) were found in all filtrates tested, but none was labelled until 142 hr., and radioactivity also appeared in many amino-acids at the same time. Examination of the dried mycelium showed that only very small amounts of carbon derived from the labelled alanine were assimilated during the first 5 days, since the counts were barely above the 'background' value of 2·2 c.p.m.; but at 142 hr. the count increased to 28 c.p.m., and at 174 hr. to 90 c.p.m.

TABLE 4

Compounds detected in the Medium Containing Glucose and ^{14}C-α-alanine during Growth Period

Time (hr.) after inoculation	Substances detected in the medium			
	Amino-acids*	Carbo-hydrates†	Phosphate esters	Organic acids
120	ser, asp, (cys)	gal, shp, cb	F6P, FDP, (R5P)	—
136	ser, asp, (cys, gly)	gal, shp, cb, (ct)	F6P, FDP, (R5P)	—
142	*ser*, asp, (cys, *gly*)	*glu*, gal, shp, *cb*	F6P, FDP, R5P, 3PGA, (G1P, G6P, R1P)	—
148	*ser*, *asp*, *gly*, (*cys*)	*glu*, gal, shp, *cb*, g-a	*F6P*, *FDP*, *R5P*, *3PGA*, *G1P*, (*G6P*, *R1P*)	malic, succinic
160	*ser*, *asp*, *gly*, (*cys*)	*glu*, gal, (shp, cb)	*F6P*, *FDP*, (*R5P*), *3PGA*, *G1P*, (*G6P*), (*R1P*)	malic, succinic
184	*ser*, *asp*, (*gly*, *cys*)	(*glu*)	*F6P*, *FDP*, *3PGA*, (G1P)	malic, succinic

* Other than alanine. † Other than unlabelled glucose.

Brackets ( ) represent trace only; *italics* indicate labelling.

Abbreviations: ser = serine; asp = aspartic acid; gly = glycine; cys = cystine; glu = glucose; gal = galactose; shp = sedoheptulose; cb = cellobiose; ct = cellotriose; g-a = glucuronic acid; F6P = fructose-6-phosphate; FDP = fructose-1,6-diphosphate; R5P = ribose-5-phosphate; R1P = ribose-1-phosphate; G6P = glucose-6-phosphate; G1P = glucose-1-phosphate; 3PGA = 3-phosphoglyceric acid.

In another series of experiments, generally labelled ^{14}C-glucose was used in conjunction with (unlabelled) α-alanine, and in this case the first amino-acid in the medium to show radioactivity was alanine.

DISCUSSION

The results given in Table 1 permit certain conclusions to be drawn concerning the metabolism of *C. globosum.* It seems that the organism is able to utilize to a varying extent the carbon of some, but not all, of the amino-acids, and of all the carboxylic acids tested, provided that a certain amount of glucose is also available. The extent to which this utilization occurs is indicated by the excess weight of mycelium produced in liquid media containing the amino- or carboxylic acids over that produced in media containing the same amount of glucose as sole source of carbon (N as nitrate). The carbon of pyruvic, lactic, or tartaric acid seems to be as easily available as that of glucose, but many amino-acids give distinctly less growth. This may be due to lower permeability of the hyphae to these compounds, or to a less ready transformation of the carbon skeleton of the amino-acids into other essential structures, or to a difficulty in supplying the amino-groups required for new syntheses. Further, the *rate* of growth varies considerably even in media which allow the attainment of the same maximum. Thus, early growth on lactate is slower than on pyruvate, possibly because the initial (? oxidative) step presents a difficulty. Further, utilization of the carbon of almost all compounds tested seems to occur only in the presence of glucose, since growth on glucose-free media was found to be negligible (exceptions being alanine and glutamic acid). This may indicate that some of the intermediate compounds formed during the complete oxidation of glucose are essential for further syntheses, e.g. as receptors of amino-groups.

It seems then that (i) transformation products derived from pyruvic, lactic, succinic, and tartaric acids are as readily available as those derived from glucose for the production of the carbon framework of the amino-acids, and (ii) many of the individual amino-acids can also be transformed into all the other amino-acids required for protein synthesis, although there are rather wide differences in the ease or completeness with which these transformations are effected. In some instances it appeared that one or more amino-acids might be formed in a quantity too great to be incorporated immediately into new protein, since chromatographic examination of the culture media on the 8th day revealed the presence of free amino-acids, notably α-alanine, aspartic acid, glutamic acid, serine, and glycine.

From a study of the sequence of appearance of various labelled compounds in the mycelium and medium, it is possible to draw certain further conclusions concerning the metabolism of α-alanine by the fungus, in a medium in which this amino-acid (containing C^{14}) forms the sole source of nitrogen, and may supplement glucose as a source of carbon. During the early stages of growth, very little of the carbon of alanine found its way into the mycelium; it seemed that almost the whole of the carbon in the 10 mg. or so of dry tissue formed during the first five days came from glucose, the dry mycelium contained 4·25 per cent. N, and to supply this not less than 2·5 mg. alanine would be needed, assuming the whole of the nitrogen to be taken up by the tissue. The fate of the non-nitrogenous (labelled) fragment left on deamination is unknown, but quite possibly it is lost by respiration. During the early period of growth various new amino-acids, sugars, &c., began to appear in the medium, but in none could any radioactivity be detected; presumably, therefore, the carbon of all these substances was derived from glucose. However, after approximately 136 hr. there was a rather sudden increase in the radioactivity shown by the mycelium, and at the same time many of the simple substances in the medium also showed activity. Evidently at this stage the carbon of alanine was being called upon for tissue building, and some of the metabolic products of the amino-acid were being produced in sufficient amount to escape into the medium.

The possibility that the closely related substances α-alanine, pyruvate, and lactate may be particularly readily transformed into 3-phosphoglyceric acid (and thence into other organic phosphates) has been suggested above. The experiments with ^{14}C-α-alanine give support to this view. In liquid media the carbon skeleton of this amino-acid was shown to contribute to the pool of organic phosphates, the amount of which in the medium reached a maximum at approximately the time when fruiting was most vigorous.

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The Role of Leaves as Inhibitors of Flower Induction in Strawberry

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With one Figure in the Text

ABSTRACT

The effects of various defoliation treatments on flower initiation were studied in the strawberry var. Talisman, which is a facultative short-day plant, with particular reference to differences in the inductive capacity of leaves of differing maturity.

Plants from which all mature leaves had been removed to leave only two immature leaves flowered in longer photoperiods than intact controls, and conversely plants bearing only three fully mature and no immature leaves required a shorter photoperiod for flower initiation than intact plants.

Intact plants in constant darkness and totally defoliated plants in continuous light both initiated flowers, but intact plants in continuous light failed to flower.

It is submitted that these results provide evidence that the photoperiodic control of flowering in this plant operates through a flower inhibitor produced in the leaves.

They also show that although leaves of any maturity are able to inhibit flower initiation, under some conditions mature leaves are more inhibitory than immature, and that the inhibitory activity of any leaf decreases with decreasing photoperiod.

INTRODUCTION

THE summer fruiting varieties of the cultivated strawberry (*Fragaria ananassa* Duch.) are perennial plants in which flower initiation occurs during the short days of autumn in September and October. The inflorescences overwinter in the bud and flower and fruit during the following summer.

It has for long been a widespread cultural practice to defoliate the plants, either totally or partially, after cropping during the course of cleaning up the plantation. In 1954 Wilson and Rogers investigated the possible cultural advantages of defoliation both by mowing and by burning off the foliage, and reported increases in cropping in the year following either of these treatments. Guttridge *et al.* (1960) have shown that increases in crop following autumn defoliation are the result of an increase in the number of flower trusses initiated, and that in some circumstances initiation may be increased several-fold by this treatment.

The work reported below was done to investigate the effect of defoliation on flower initiation in the strawberry, and in particular to look for differences in the capacity of leaves of differing maturity to promote flower initiation.

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Earlier work by Guttridge (1959 *a* and *b*) has provided evidence that the photoperiodic responses of the strawberry are controlled by a hormone, produced in the leaves in long daylengths, which promotes vegetative growth and inhibits flower initiation. It was thought possible that enhancement of flower initiation following defoliation might be due to the removal of the site of production of this hormone in the mature leaves.

MATERIALS AND METHODS

Plants of the variety Talisman were used throughout this series of experiments. At the temperatures used this variety behaves like a short day plant with a critical daylength of about 14 hours. Flowers are formed terminally and growth is continued by the bud in the axil of the last leaf below the flower. The plants were restricted to single crowns so that only one inflorescence could form at a time on any plant. Quantitative measurements of the degree of induction were made by one or more of the following methods: (*a*) counting the number of leaves produced between the start of treatment and initiation; (*b*) the number of short day cycles required for induction; and (*c*) the proportion of plants initiating flowers during treatment. The plants were dissected soon after the end of treatment to find if flowers had been initiated.

The plants were grown in non-inductive conditions before the start of treatment. In summer these were provided by the natural long summer days and in the winter by a light-break from incandescent tungsten lamps of 4 hours' duration during the night. For the duration of treatment in Expts. 2, 3, and 4 they were grown in four similar growth cabinets in which light of approximately 1,000 f.c. at the leaf surface was provided by ten 80-watt and four 40-watt Warm White fluorescent tubes, supplemented with one 75-watt and two 100-watt incandescent lamps suspended 18 in. above the plants, behind a screen of two layers of window glass. After treatment, except in Expt. 4, they were returned to the glasshouse and grown on with a nightly light-break until dissected.

At the start of treatment there were about seven fully expanded leaves on each plant, except in Expt. 4 when there were about ten. The treatments used were as follows; not all were applied in every experiment:

1. Plants left intact.

2. All mature leaves removed to leave only the youngest fully expanded leaf and the emergent leaf. The plants were maintained in this condition till the end of the experiment by successively removing the oldest leaf present as younger leaves became fully expanded.

3. All immature leaves removed to leave only three mature leaves; these were usually the fourth, fifth, or sixth in order of age at the beginning of treatment. All leaves emerging during treatment were removed before they had started to unfold.

4. The fourth, fifth, and sixth leaves were retained as in (3) and each younger leaf was partially defoliated by the removal of two of its three

leaflets. As further leaves emerged they too were partially defoliated before they had unfolded. This treatment was used only in Expt. 1.

5. Plants totally defoliated close to the crown; leaves emerging after treatment were allowed to develop. The totally defoliated condition lasted about one week, after which the first new leaf to emerge began to unfold.

RESULTS

Experiment 1. Young runners were potted up from the field in summer and grown outside until August 15, 1957, when treatments were started. The plants were grown outside under natural conditions of daylength and temperature throughout the experiment.

The following treatments were used: (1) intact plants, (3) three mature leaves only retained, (5) all mature leaves removed; and (4) three mature leaves retained with younger leaves reduced by two-thirds. Each treatment contained thirty plants.

In pre-treatment dissections flower trusses were found after the fourth or fifth leaf in the bud in ten out of twenty-five plants dissected. These flower trusses, which had probably been formed as a result of the disturbance following removal from the field and potting up, did not interfere with future initiation and provided an additional reference-point when computing the rate of growth of the plants during the experiment. Fifteen of the thirty plants in each treatment were dissected on September 25 after 7 weeks and the remainder on December 13.

The results of the two dissections have been combined and are shown in Fig. 1.

The plants from which all mature leaves had been removed flowered at the same time and to the same degree as the intact controls. Plants defoliated to three mature leaves had not initiated flowers at the time of the first dissection, and eventually formed flowers about two plastochrons later than the controls. Plants with three mature leaves and one-third of each younger leaf occupied an intermediate position between the two groups, initiation having been delayed by about one plastochron.

The removal of mature leaves had no effect on flowering. This shows that the mature leaves are relatively non-promotive even, as in this case, under conditions extremely favourable to flower initiation. Plants with only one fully expanded leaf appeared to be as efficient in the initiation of flowers as intact plants. On the other hand, the removal of all young leaves or, in the intermediate treatment, the removal of two-thirds of each young leaf caused a delay in flower initiation. It may be argued, either, that in the natural short days of autumn all leaves promote flower initiation but that mature leaves are less effective than immature, or, that neither mature nor young leaves entirely inhibit flower initiation under these conditions, although mature leaves are more inhibitory than immature. Retention of one-third of all young leaves in plants otherwise defoliated to three mature leaves promoted flower initiation; either, by increasing the area of effective tissue present, or, by competing

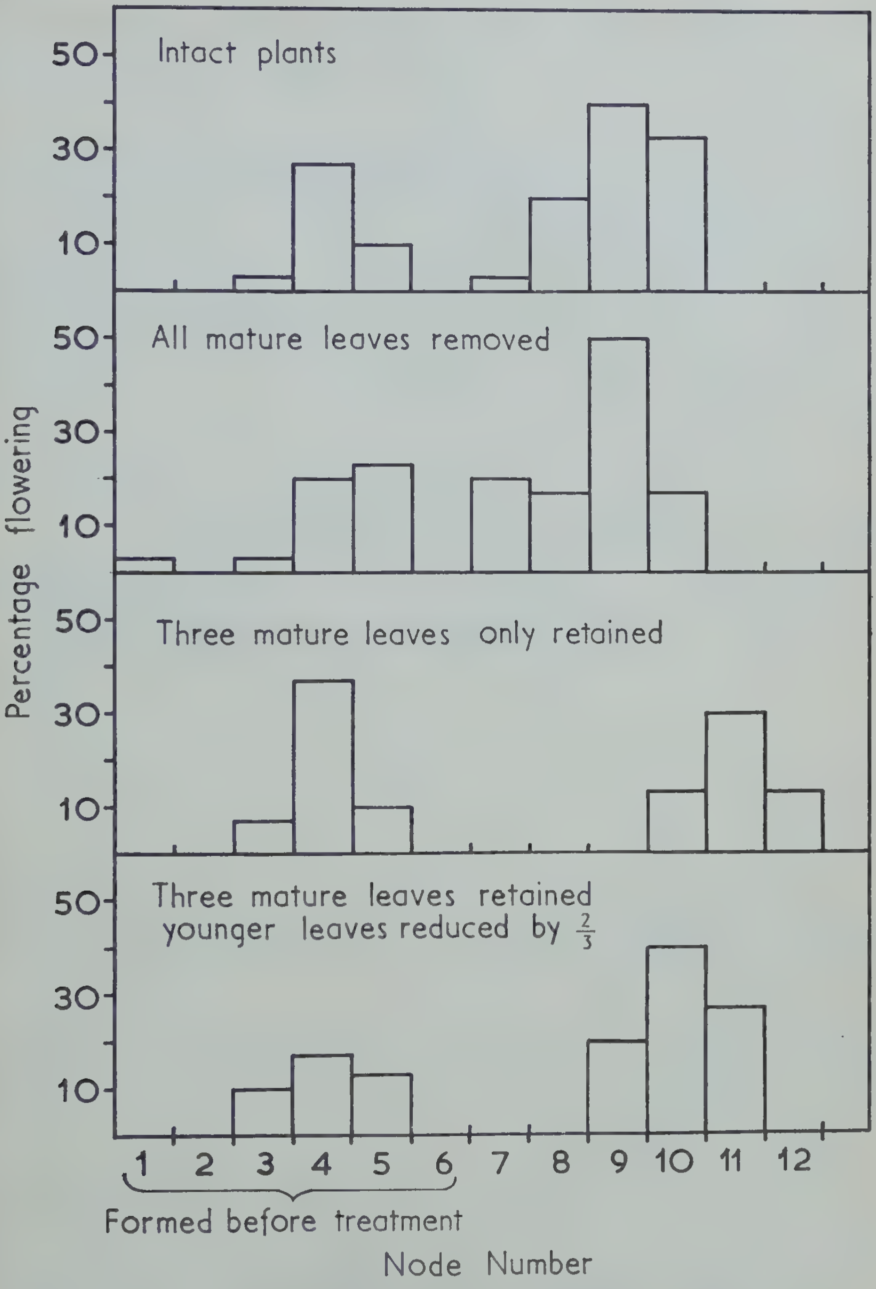

FIG. 1. Percentage of plants bearing flowers at different nodes.

with the growing-point for the hormone produced in mature tissues. Two experiments were set up to test these possibilities.

Experiment 2. Plants from which all mature leaves had been removed were compared to intact plants. Both groups were grown together under a range of daylengths to see whether the removal of old leaves led to a promotion of flower initiation, as would be expected if their presence was actively inhibitory to it.

Runners were potted from the field in August and kept outside till early September, when they were moved into a warm glasshouse and grown on with a light-break during the night. On October 2 they were transferred to four similar growth cabinets providing daylengths of 10, 12, 14, or 16 hours at a temperature of 60°F. Thirty-six plants were put in each cabinet, sixteen of which were defoliated to the youngest fully expanded leaf; the other twenty plants were left intact. Four plants of each treatment were removed at 2-day intervals, and returned to the glasshouse for 3 weeks before dissection. The results of this dissection are shown in Table 1.

TABLE 1

Number of Plants with Flowers in samples of Four Plants after Varying Numbers of Photoperiodic Cycles

Number of days in cabinets	Intact plants Daylength (hrs.)				All mature leaves removed Daylength (hrs.)			
	10	12	14	16	10	12	14	16
8	3	2	—	—	4	4	—	—
10	4	4	1	—	4	4	4	—
12	4	4	2	0	4	4	4	1
14	4	4	3	0	4	4	4	2
16	4	4	0	0	—	—	3	3
18	—	—	—	0	—	—	—	1
20	—	—	0	—				
24	—	—	—	0				

Plants from which mature leaves had been removed initiated flowers regularly in a 14-hour daylength and had a critical daylength of about 16 hours in which flowering was rather irregular. Intact plants failed altogether to initiate flowers in a 16-hour daylength.

These results show that removal of the mature leaves raised the critical photoperiod for flower initiation, and, since young leaves were present in both treatments, support the suggestion that mature leaves are actively inhibitory to flower initiation.

Experiment 3. Plants which had been cold-stored to prolong dormancy were potted in June and after growing on outside were transferred to four similar growth cabinets on August 14, in which they were exposed to daylengths of 10, 12, 14, or 16 hours at 60°F. Thirty-six plants in each cabinet were divided into four treatments each of nine plants as follows: intact controls; mature leaves removed; three mature leaves only; totally defoliated. The plants were kept in the cabinets for thirty-six days, after which they were transferred to the glasshouse and grown on for a further 3 weeks before dissection.

Table 2 shows the number of plants with flowers for each treatment for each daylength.

TABLE 2

Number of Plants with Flower Buds after Different Daylength Treatments

Treatments	No. of flowers present in daylengths of			
	10 hrs.	12 hrs.	14 hrs.	16 hrs.
Three mature leaves only . .	9	7	0	0
Intact plants	9	9	2	0
No mature leaves (young leaves present)	9	9	9	1
Defoliated	8	9	9	8

Neither intact plants nor plants bearing only mature leaves initiated flowers freely when the daylength was longer than 12 hours. Plants from which all mature leaves had been removed initiated flowers at 14 but not at 16 hours, but totally defoliated plants were able to initiate flowers freely at all the daylengths used. The presence of either young or old leaves reduced the critical daylength for induction. This suggests that flowering might be entirely controlled by an inhibiting system produced in the leaves, which is especially active in long daylengths. If this is the case plants should be able to initiate flowers in total darkness, or, if totally defoliated, in continuous light. Exp. 4 was done to examine these possibilities.

Experiment 4. Talisman plants which had been growing in the glasshouse with a light-break of 4 hours during the night were transferred on November 5 to four growth cabinets. Two of the cabinets were maintained at 60°F. and two at 65°F. At each temperature one cabinet was illuminated continuously, the other was kept in darkness. Thirty-six plants were put into each cabinet; half were totally defoliated and half left intact. Five plants of each treatment were removed and dissected for the presence of flowers after 11 days in the cabinets, a further five after 18 days, and the remaining eight after 28 days.

The dark cabinets had to be opened daily for a few minutes for watering; at this time external light was kept to a minimum. Measurements showed that, in the corner of the cabinet nearest to the source of light, the intensity was slightly less than one foot candle and very much less than this in other parts of the cabinets.

The results of the experiments are shown in Table 3. Plants which were totally defoliated and grown in the dark suffered severely from *Botrytis cinerea* infection and as only three were still alive at the time of the final dissection this treatment has been omitted from the table of results. No differences in response were found at the different temperatures used, and the results of the two temperature treatments have been combined in Table 3.

Plants in all treatments initiated flowers except for intact plants kept in continuous light. This is the result which would be expected if flower

TABLE 3

Number of Flowers Initiated by the Time of Dissection in Experiment 4.

Treatment	Dissected after 11 days (10)*	 18 days (10)*	 28 days (16)*
Intact in light .	0	0	0
Defoliated in light .	1	3	13 (13)*
Intact in darkness .	1	2	8 (15)*

* Number in brackets refers to number of plants dissected on each occasion.

induction was not dependent upon a flower-promoting substance, but was controlled entirely by an inhibitor produced in the leaves. Flowering was not confined to the first leaf position to differentiate after entry to the cabinets and so cannot be explained as the result of the completion of a partial induction initiated before the start of treatment.

DISCUSSION

It is submitted that the experimental results reported above can be explained most simply in terms of the regulation of flower initiation by means of an inhibitory system or substance produced in 'non-inductive' daylengths.

However, the results do not entirely exclude the possibility that, in addition, flower initiation may depend on the presence of a flower-promoting factor. First, because although totally defoliated plants were able to initiate flowers, they may still have been able to use stored products for the metabolism of a flower-promoting substance. Thus Lona reported flower initiation in two short-day plants, *Perilla nankinensis* (1950 *a* and *b*) and *Chenopodium amaranticolor* (1948), when grown in total darkness, but in both cases found that young plants with smaller carbohydrate reserves were unable to form flowers. Secondly, because the totally defoliated condition was not maintained for the duration of the experiment, unfolding leaves again being present after about 1 week, and in the interval small stubs of green tissue remained on the plants. Nevertheless, in this treatment flowers were formed in all photoperiods and in continuous light, so that if a flower-promoting substance was formed its production would appear to be independent of photoperiodic control. Thirdly, because in the continuous-darkness treatment the plants were in fact exposed to measurable light for a brief interval on most days. Harder and Gummer (1947) have shown that although *Kalanchoe blossfeldiana* will not produce flowers in total darkness a daily illumination lasting for only 1 second is sufficient to promote flowering. Schwabe (1959) has confirmed this result. As far as the comparison can be made the total amount of incident light on the plants in the experiment described above was, at its brightest, approximately equal to that provided by Schwabe, but in the whole of one cabinet and most of the other it was far below this level. The flowering response showed no sign of being altered by these variations in the amount of light.

If indeed a flower-promoting substance is present in the strawberry, it appears to be able to act independently of photoperiod and cannot, therefore, be the agent through which photoperiodic control of flowering initiation is exercised.

The results of these experiments may be interpreted as showing that the strawberry apex is able to produce flowers under any conditions of photoperiod but is prevented from doing so in long days by the action of an inhibitor and that this inhibitor is a hormone formed in the leaves. These results are in agreement with, and extend, those of Guttridge (1959 *a* and *b*), who used pairs of runner plants, joined by the stolon, growing in different photoperiods. He reported that a runner plant growing in short days was prevented from forming flower trusses when joined to a runner growing in long days and suggested that this inhibition was due to the activity of a hormone, produced in the leaf in long days and translocated thence to the plant in short days, where it promoted vegetative growth and inhibited flower initiation at the apical meristem. Thompson and Guttridge (1959) have shown that the activity of the naturally produced hormone may be simulated by applying gibberellic acid to strawberry plants, and have suggested that the hormone may be a gibberellin-like substance.

The experiments reported above provide additional information on the activity and production of this hormone. Thus it would appear: (*a*) *that leaf tissue of any age can inhibit flower initiation*: this is illustrated in Exp. 3 in which the presence of either immature or mature leaves reduced the critical photoperiod when compared to totally defoliated plants. In Expt. 4 it was found that totally defoliated plants were, in fact, able to flower in continuous light; (*b*) *that the inhibitory activity of any leaf is related to photoperiod*: in all treatments the degree of flower inhibition fell when the photoperiod was reduced; (*c*) *that mature leaves are more actively inhibitory than immature to flower initiation*: this is clearly demonstrated in Expt. 3, in which immature leaves inhibited initiation only in a 16-hr. photoperiod, while mature leaves were inhibitory in both 14- and 16-hr. photoperiods.

However, the effect of the presence of both immature and mature leaves in intact plants is not additive, and young leaves were always found to reduce the inhibitory effect of mature leaves. Thus intact plants initiated flowers sooner and in longer daylengths than did plants reduced to mature leaves only. The experiments of Guttridge, cited earlier, with runner pairs provide evidence that the postulated hormone is a vegetative-growth promoter as well as a flower inhibitor and is transferable in the carbohydrate stream to tissues remote from its source. It seems likely that young actively expanding tissues may have a high requirement for the hormone and may act as a 'sink' for hormone produced in more mature leaves. Additional evidence for this view is provided by Expt. 1, in which some plants had two-thirds of each immature leaf removed before it had started to unfold. These plants occupied a position midway between intact plants and plants defoliated to three mature leaves. This is the result which would have been expected if the immature tissues acted as 'sinks', and if their activity as 'sinks' was proportional to their volume.

It must be recognized that in defoliated systems such as the ones used in these experiments, the plants are subjected to gross interference in their natural balance of leaf tissue, and considerable metabolic disturbances must be expected. For instance it seems likely that when the plant becomes wholly dependent on immature leaves, following the removal of all mature leaves, meristematic demands have to be satisfied by a precocious and more considerable drain from these tissues than would normally be the case.

Thus although, under experimental conditions involving the removal of all mature leaves, immature leaves were found to be inhibitory to flower initiation in daylengths of 16 hours or longer, it seems reasonable to suppose that in intact plants, under natural conditions, immature tissues would contribute little or nothing to the level of inhibitor at the apical meristem. Similarly when plants are defoliated to three mature leaves the apical meristem would receive an unnaturally high proportion of metabolites from leaves of this age-group. Although these considerations do not in any way weaken the main argument in favour of the control of flowering through a growth-promoting and flower-inhibiting hormone, they must be borne in mind when using defoliation techniques as a method of assessing the activity of leaves of different ages on flower initiation.

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Methods of Estimating the Leaf Area of Grasses from Linear Measurements

BY

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ABSTRACT

This paper is concerned only with estimates of leaf area obtained from linear measurements on the leaves. Past investigations on grasses and on other species are briefly reviewed. Various weighted regressions are examined, the object being to obtain a satisfactory estimate of the constant k in the prediction equation $A = kLB$ (L = length of leaf; B = breadth at a point midway along the length; A = area). Sets of data from four different grasses are examined. The formula $A = 0{\cdot}905LB$ is consistent with all these data. The practical use of the technique is discussed.

INTRODUCTION

THE area of a grass leaf is particularly difficult to measure in any reasonably direct way (e.g. by planimeter) because of the narrowness of the blade and, in general, its small size. It is therefore natural that indirect methods of estimation have been sought. Apparatus such as Jenkins's (1959) airflow planimeter or photo-electric devices tend to be rather expensive. Methods utilizing relationships between leaf weight and leaf area have been widely used, but in this paper we are concerned only with estimates obtained from linear measurements made on the leaves. The object has been to select a technique which is fairly rapid, is not necessarily destructive of the leaves, does not need elaborate equipment, and ultimately requires only a very simple calculation to give the estimated area.

PAST INVESTIGATIONS ON VARIOUS SPECIES

The idea of utilizing a combination of linear measurements is an obvious one and has been used for many species of plant. In choosing which measurements to take, various authors have considered the geometry of the leaves of the species. Freeman and Bolas (1956), for example, found that the apple leaf approximates closely to an ellipse; hence, using the usual formula for the area of an ellipse in terms of its axes, they were able to construct a grid which gave a direct reading of area. This proved to be a rapid and efficient method for use in the field. On the other hand, Owen (1957) constructed a grid for sugar-beet leaves using the empirical finding that there is a linear relation between area and the product of a conveniently chosen pair of measurements.

The grass (or cereal) leaf is not very closely approximated by any simple

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geometrical shape, but it is plausible to regard it (as Hopkins (1939) did the wheat leaf) as a trapezium with a small isosceles triangle on top. Studying this model, one might anticipate the existence of a fairly close relationship between area and the product of length and breadth (the latter being measured midway along the length).

A number of authors used the idea of determining a constant k in the equation

$$A = kLB. \tag{1}$$

Clements and Goldsmith (1924), using wheat and oats, determined an average k† from a sample of n leaves as follows:

$$k = \frac{1}{n}\sum\left(\frac{A}{LB}\right), \tag{2}$$

where L and B are the extreme length and breadth.

Lal and Subba Rao (1950 *a*, *b*, 1951) for various cereals determined $1/k$ thus:

$$\frac{1}{k} = \frac{1}{n}\sum\left(\frac{LB}{A}\right); \tag{3}$$

here L is extreme length and B is the breadth at mid-length (this latter definition of B is used in all subsequent parts of this paper). They suggested using the value of k so determined for future estimations of leaf area as follows:

$$\log A = \log L + \log B + \log k, \tag{4}$$

and for the total leaf area of a new sample of N leaves they suggested

$$\log \sum A = \log \sum L + \log \sum B + \log k - \log N. \tag{5}$$

Formula (5), however, gives a biased result which A. W. Kemp showed could only be corrected by taking into account the covariance between L and B (see Langer (1956)). Neither Clements and Goldsmith nor Lal and Subba Rao discussed statistical assumptions and validity.

Hopkins (1939) adopted a somewhat different approach to the area of wheat leaves. He estimated regressions of the form

$$\log A = \alpha \log L + \beta \log B + \gamma. \tag{6}$$

He investigated the value of taking an additional measurement of breadth at three-quarters of the distance from base to tip. He also dealt with the statistical aspects of the estimation procedure in some detail.

In the past, the methods both of Lal and Subba Rao, and of Hopkins have been used and modified for grasses at this Institute (Langer (1956)). Measurements of breadth at the quarter and three-quarter lengths have been examined in addition to B, but on the whole, it seems that the additional effort involved in taking more than one breadth measurement per leaf is not worth while (particularly since the emphasis has been on devising a rapid technique).

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† To avoid confusion, all formulae of other authors will be given in our notation, i.e. that of equation (1).

It has been found that when equations of the form (6) are fitted to data from grass leaves, both α and β tend to be very close to unity. Clearly, considerably less computational effort would be required if one could assume that $\alpha = \beta = 1$. It would then, of course, be desirable to estimate γ subject to this assumption.

Recently, relatively large amounts of suitable data have been collected at Hurley, so the comparison of different simple weighted-regression models was undertaken.

THE REGRESSION MODELS STUDIED

If LB is plotted against A, it is usually found that the scatter of the points tends to increase with increasing area. To allow for this, some system of weighting is desirable, the principle being that the weight (i.e. the relative importance) given to any point when calculating the regression is inversely proportional to the variance at that point.

The logarithmic regression (6) has the property of automatically weighting the data—it is roughly equivalent to using weights on the original data inversely proportional to the square of the area. However, if we assume $\alpha = \beta = 1$, we can deal with a single variable (LB), and this allows us to dispense with logarithms (whose main advantage, apart from weighting, was computational ease). We can then examine more than one system of weighting.

The Form of the Regression

For notational convenience let

$$Y \equiv LB. \tag{7}$$

The fact that $\alpha \doteqdot 1 \doteqdot \beta$ in the logarithmic regressions suggests that the (A, Y) relationship will be approximately linear. Again, physical considerations indicate that it is sensible to assume that the regression line passes through the origin ($A = 0$, $Y = 0$).

If, for the data available, Y is plotted on graphs against A, the reasonableness of these assumptions is confirmed. The increase in scatter, noted in previous data, is also apparent, but it is not possible to determine by inspection whether the scatter is any particular function of the area.

It was necessary to decide whether to regress A on Y or Y on A. There are good arguments for either procedure. We decided to regress Y on A because it seemed likely that errors of measurement in the data studied were greater in Y than in A. (It is a usual assumption in regression analysis that the 'dependent' variate is subject to error, but the 'independent' variate is not.) A resultant advantage is easier computing because it is in general more convenient to use weighting systems based on the 'independent' variate, and in this case it is more logical to relate the weighting system to A. Also Y might be said to 'depend' on A, since the latter has a more direct meaning.

The Weighting Systems

Three simple systems of weighting were adopted:

(i) Unweighted.
(ii) Weight inversely proportional to area.
(iii) Weight inversely proportional to the square of the area.

System (i) corresponds to the usual regression situation, i.e. the absolute error about the regression line is effectively constant over the range of values being studied; (iii) assumes that the relative error (analogous to the coefficient of variation) is effectively constant; (ii) is an intermediate situation with absolute error increasing but relative error decreasing with increasing area.

For each system we obtain estimates b, s_b, and s^2 of a regression coefficient β, its standard error σ_β, and the residual (weighted) variation σ^2 about the line. It is also easy to find the proportion of the total (weighted) variation accounted for by the regression.

Statistical Derivation

We assume a general model of the form

$$Y_i = \beta A_i + \epsilon_i; \qquad \epsilon_i = N(0, \sigma_i^2); \qquad i = 1, 2, \ldots, n \tag{8}$$

(i.e. the ϵ_i are distributed independently as a normal distribution, mean 0, variance σ_i^2).

The three systems of weighting correspond to:

$$\left.\begin{array}{rl} \text{(i)} & \sigma_i^2 = \sigma^2 \\ \text{(ii)} & \sigma_i^2 = A_i\,\sigma^2 \\ \text{(iii)} & \sigma_i^2 = A_i^2\,\sigma^2 \end{array}\right\}. \tag{9}$$

We adopt Deming's (1943) definition that the weight of a function f is inversely proportional to its variance:

$$w_f = \frac{\sigma^2}{\sigma_f^2}; \tag{10}$$

σ^2 is a proportionality factor and is the variance of a function of unit weight.

Hence the three weighting systems are

$$\left.\begin{array}{rl} \text{(i)} & w_i = 1 \\ \text{(ii)} & w_i = 1/A_i \\ \text{(iii)} & w_i = 1/A_i^2 \end{array}\right\}. \tag{11}$$

To obtain the least squares estimates of b, s_b, and s^2, we minimize

$$\sum_{i=1}^{n} w_i(Y_i - bA_i)^2$$

in the usual way. This leads to

$$\left.\begin{aligned} b &= \frac{\sum wAY}{\sum wA^2} \\ s^2 &= \frac{1}{n-1}\left[\sum wY^2 - \frac{(\sum wAY)^2}{\sum wA^2}\right] \\ s_b &= s/\sqrt{(\sum wA^2)} \end{aligned}\right\}. \tag{12}$$

For example, the three regression coefficients are

$$\left.\begin{aligned} b_{(\mathrm{i})} &= \frac{\sum AY}{\sum A^2} \\ b_{(\mathrm{ii})} &= \frac{\sum Y}{\sum A} \\ b_{(\mathrm{iii})} &= \frac{1}{n}\sum\left(\frac{Y}{A}\right) \end{aligned}\right\}. \tag{13}$$

It is interesting to note that $b_{(\mathrm{iii})}$ is Lal and Subba Rao's estimator. We also note that we should obtain the same estimation equation either by regressing Y on A with $w = 1/A$ or by regressing A on Y with $w = 1/Y$.

Lastly, the percentage of the total weighted variation in Y accounted for by regression is

$$100 \times \frac{(\sum wAY)^2}{(\sum wA^2)(\sum wY^2)}. \tag{14}$$

Discriminating between the Models

Some difficulty arises when, given a set of experimental data, one wishes to decide which model is most appropriate. The method we adopted was to find the median value of A (say A_m) for the set and then to divide the data into two groups P and Q: in P, all $A \leqslant A_m$, while in Q, all $A \geqslant A_m$. The calculations for b, s_b, and s^2 were made separately for each group. If the weighting is appropriate, the value of s^2 should be approximately the same for each group. It is also desirable to check that there is no suggestion of systematic (weighted) variation about the line—this can be carried out graphically. This method of grouping also gives some check on the linearity hypothesis by enabling one to compare the two values of each type of b.

DATA AND RESULTS

Description of data. Two main series and two subsidiary series of data were examined. It must be emphasized that they refer to uninterrupted growth for varying periods from the start of the season; i.e. no data on regrowths were available. The two main series comprised 120 leaves each of S.23 perennial ryegrass (*Lolium perenne*) and S.37 cocksfoot (*Dactylis glomerata*). Each set of 120 was made up of 6 groups of 20 leaves, one group being taken at each of 6 sampling dates (at fortnightly intervals). The remaining series comprised

45 leaves of S.215 meadow fescue (*Festuca pratensis*) and 40 leaves of S.48 timothy (*Phleum pratense*).

All leaves were carefully blue-printed and the measurements analysed were taken from the blue-prints. Area was measured by counting millimetre squares on a grid superimposed upon the print. Experience at Hurley is that this method, although tedious, is more reliable than the use of a planimeter (for grass leaves). All area measurements were made by at least two independent observers. Measurements of length and breadth were made by using a sheet of millimetre graph paper as a ruler. Length measurements were to the nearest millimetre, and those for breadth to the nearest 0·5 mm.

The ranges of leaf area, length, and breadth for the four sets of data are given in Table 1.

TABLE 1

Ranges of Values of the Data Examined

	No. of leaves	Leaf area (A mm.^{2})	Length (L mm.)	Breadth (B mm.)
S.23 Perennial ryegrass . .	120	8–389	10–167	1·0–3·0
S.37 Cocksfoot . . .	120	26–1,179	13–223	1·0–7·0
S.215 Meadow fescue . .	45	125–2,678	43–316	2·5–10·5
S.48 Timothy	40	68–1,212	25–246	2·5–6·0

It can be seen that the range of leaf areas examined was considerable (from 8 mm.^{2} to 2,678 mm.^{2}). The basic shape of the leaves differed considerably between species, e.g. the ratio of length to breadth is approximately twice as large for ryegrass as for cocksfoot leaves. Also within each of these species this ratio tended to increase with sampling date, but this probably occurred because leaf area was increasing and, within a species, larger leaves tend to have a higher $L:B$ ratio.

Results. Preliminary examination of the ryegrass and of the cocksfoot data indicated that within each species the relationship between A and Y was consistent for all 6 dates. The 120 leaves were therefore considered as a whole, ranked according to their area and split into two groups, P and Q, by the median area, as described above.

Formulae (12) and (14) were then applied to both P and Q and to the complete set of leaves, assuming each of the three models (formulae (11)) in turn.

Detailed results for S.23 perennial ryegrass are given in Table 2.

It is apparent that the estimate b of the regression coefficient is substantially the same for each model and for both groups P and Q.

To decide which model is most appropriate for these data, we adopt the criterion mentioned earlier: that s^2 should be approximately the same for both groups. We find that either model (ii) or model (iii) fits reasonably well and this suggests that the true situation lies somewhere between them. A possible explanation is that the basic relationship is close to (iii), but that errors of measurement in L and particularly in B are relatively much larger for the

TABLE 2

Results for S.23 Perennial Ryegrass

120 leaves split into two groups: P ($7 < A < 108$) and Q ($108 < A < 390$)

Model	$w = 1$	$w = 1/A$	$w = 1/A^2$
	Regression coefficient (b)		
Group P	1·1065	1·1054	1·1031
Group Q	1·1123	1·1100	1·1088
All data	1·1118	1·1089	1·1059
	Estimated standard error of b (s_b)		
P	0·01400	0·01457	0·01544
Q	0·00974	0·01087	0·01210
All data	0·00716	0·00834	0·00977
	Percentage (weighted) variation accounted for by regression		
P	99·07	98·98	98·86
Q	99·55	99·44	99·30
All data	99·51	99·33	99·08
	Estimated residual (weighted) variation (s^2)		
P	52·2	0·783	0·01430
Q	275·0	1·439	0·00879
All data	162·3	1·102	0·01147

smaller leaves of the set—this effect is likely to be particularly marked in the ryegrass results, since the breadths of leaves varied only from 1 mm. to 3 mm. and the unit of change was 0·5 mm. This effect of errors of measurement is perhaps a reason for the slow fall in the coefficient of variation as the mean increases, which is frequently found in biological data. Since cocksfoot leaves tend to be both larger and also broader in relation to their length, one would expect that errors in B would have less influence and, in fact, the cocksfoot data show a closer agreement with model (iii).

TABLE 3

Summary of Results for Four Species

	b	s_b	s^2	% variation accounted for
	Model (iii): $w = 1/A^2$			
S.23	1·1059	0·00977	0·0115	99·1
S.37	1·1043	0·00652	0·00509	99·6
S.215	1·103	0·0119	0·00639	99·5
S.48	1·107	0·0168	0·0112	99·1
	Model (ii): $w = 1/A$			
S.23	1·1089	0·00834	1·102	99·3
S.37	1·1051	0·00589	1·172	99·7
S.215	1·120	0·0107	5·776	99·6
S.48	1·094	0·0167	4·944	99·1

Examining Table 2, it is interesting to note that model (i) has given both the lowest estimated s_b and the highest value of percentage variation accounted for by regression. This suggests that neither of these would be a satisfactory test of goodness of fit, since we know that model (i) is not really appropriate.

This illustrates a general point: the validity of any result is dependent upon the correctness of the assumptions (explicit and implicit) made in setting up the analysis.

Table 3 summarizes the results for all four grasses based on models (ii) and (iii).

The striking thing about this table is the very close agreement between the estimates b for all four species and for both models. It is clear that if we adopt model (iii), $LB = 1{\cdot}105A$ would adequately describe all the data examined and that 1·105 would also be a perfectly satisfactory estimate of β for model (ii). Hence for estimation we suggest using the equation

$$A = 0{\cdot}905LB. \tag{15}$$

For contrast, Table 4 shows the corresponding logarithmic regressions. No such similarity in the values of the constants for the different species is apparent, yet over quite wide ranges they lead to very similar estimates of A for given pairs of L and B. This apparent lack of similarity is due to the close interrelationships between the three coefficients within any one logarithmic regression.

TABLE 4

Estimates of Constants in Logarithmic Regressions

$$\log A = \alpha \log L + \beta \log B - \gamma$$

	α	β	γ
S.23	1·0352	0·8537	0·0627
S.37	1·0066	0·9767	0·0400
S.215	1·0581	0·7926	0·0081
S.48	1·0112	1·0245	0·0793

Finally, it is worth noting that although in Table 3 the percentage variation accounted for by regression is very high for all species (it would correspond in the simple unweighted case to a correlation coefficient of about 0·99) the residual variation about the regression line is still of the order of 5–10 per cent. (model (iii)).

DISCUSSION

When indirect techniques of this type are used in experimental work, it is vital that the regression constant shall be effectively the same for all treatments, or else conclusions based on an analysis of the estimated values may be misleading. The close agreement which we have found between the estimates of β for the different species and for the size groups within a species is very encouraging. The leaf shapes varied considerably and the range of sizes was very large. However, all these data are from hay-type managements and it would be desirable to make a sample check of the constancy of b in any particular type of experiment.

Although all results presented here are based on blue-print measurements, no additional difficulties arise when L and B are measured on actual leaves. Blue-prints were a convenient way of ensuring that individual measurements could be repeated, or additional ones taken, at any time.

Considerable care is required in measuring B; the use of an illuminated magnifying scale is recommended, as this enables one to read to 0·1 mm. Recent comparisons between measurements made with such an instrument and those made with a simple rule (to 0·5 mm.) suggest that there may be a very slight systematic difference between them. It is thought that this is due to a tendency of our observers to be slightly optimistic when estimating the nearest 0·5 mm. (i.e. if in doubt they apparently prefer to round upwards). Since all our results are based on simple-rule measurements, the use of an illuminated scale might lead to a slightly higher estimate of β than that obtained here. However, within any particular experiment, the absolute value of β is relatively unimportant; it is its constancy over all treatments that really matters.

With a relationship as close as we have found here, any of the three models would lead to a satisfactory estimate of β. It is unlikely that it will be necessary in most practical situations to specify the model. Usually, the area of a sample of leaves (say 50) from each of a number of experimental units (e.g. plots) will be estimated and these areas will be analysed as if they were the actual areas. Provided β is constant, the errors introduced by the estimation technique will be analogous to sampling errors and will have the effect of increasing the error term in the analysis. If it is felt that some transformation of the data is required, it should usually be adequate to transform the values of the area which are actually being analysed (e.g. estimated leaf area of a plot).

ACKNOWLEDGEMENTS

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The Structure and Development of Vascular Nodules in Apple Bud-unions

BY

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With two Plates and two Figures in the Text

ABSTRACT

An account is given of the development of new meristematic centres (vascular nodules) initiated in callus tissue formed in apple stems as a result of budding. Both amphivasal (xylem peripheral) and amphicribral (phloem peripheral) nodules occurred, and attention is drawn to the relationship between the type of nodule formed and the tissue in which it originated. The similarity of these structures to the meristematic nodules arising in tissue cultures is discussed, and their possible practical significance as an aid in the rooting of cuttings is pointed out.

INTRODUCTION

THE initiation of new cambial meristems in callus tissue is of vital importance in horticultural practice since it is essential for establishing vascular continuity in grafted plants and plays a part in the rooting of cuttings. Irregularly located cambia often arise in wound tissues, and the more fundamental aspects of cambial initiation have been investigated in tissue culture where they frequently originate in the shape of spherical nodules. In his review of wound healing in higher plants Bloch (1952) has stressed the complementary nature of the two lines of investigation. In tissue cultures Morel (1948), Ball (1950), and Gautheret (1955, 1957) have described the development of vascular nodules; Steward, Mapes, and Mears (1958) recently demonstrated the growth of complete carrot plants from amphicribral nodules. In the wound callus of growing plants various types of meristematic islands and bundles have been described by Küster (1925). Sass (1932) briefly records the presence of meristematic islands with woody centres described as 'callus knots' in the overhanging lip of apple graft-unions, and Sylwester and Countryman (1933) followed their development in the bark callus. Scaramuzzi (1952) has described 'lignified nodules' that occur in the callus of peach bud-unions, and Mosse (1953) observed meristematic nodules with peripheral xylem in the xylem-derived callus of ring-grafted apple-trees, and believed that sectors of normally organized stem tissue occasionally arose from them. More detailed observations on the development of vascular nodules were made

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during a recent study of callus development in young apple bud-unions where they occurred with some regularity, especially in one of the stock/scion combinations examined. Two types of nodules were found, differing in the relative positions of xylem and phloem, i.e. amphicribral (phloem peripheral) and amphivasal (xylem peripheral). A study of their origin showed a clear relationship between the type of nodule formed and the tissue from which it originated, and gives some information about the factors determining the direction of cambial activity.

MATERIALS AND METHODS

Two sets of material were examined. The first set consisted of the two-year-old apple rootstocks M. II, M. VII, and M. 26, budded in August 1958 with the variety Fortune. In August 1959 a similar set was budded, but only two rootstocks M. II and M. VII were included, and in addition there were two extra treatments intended to find out the effect of the bud upon callus formation. In one of these the bud was replaced by a similarly shaped piece of plastic material, in the other no bud was inserted. Normal budding practice was followed. Essentially this consists of making a T-shaped slit in the bark of the stock, and inserting a dormant bud, cut with a small shield of stem tissues taken from a 4-month-old shoot of the scion variety. In order to provide extra material two buds were inserted one above the other at 5 in. and 7 in. above ground level respectively. They were tied in with strips of a plastic material, and lightly covered with Vaseline.

Bud-unions from two trees of each rootstock-scion combination in the 1958 set were examined 3, 4, 8, 12, and 20 weeks after budding. The material was fixed in formalin-acetic-alcohol, and longitudinal and transverse sections, cut by sledge microtome, were stained with safranin and fast green to show their general anatomical structure, and with resorcin blue to show the location of any callose deposits (Cheadle, Gifford, and Esau, 1953). The same procedure was followed with the material budded in 1959 except that the samples were collected earlier—1, 2, 4, 8, and 16 days after budding.

It is perhaps relevant to point out that the buds do not start to grow till the following spring, after the rootstock has been cut back. At the time of examination the rootstock top was still intact.

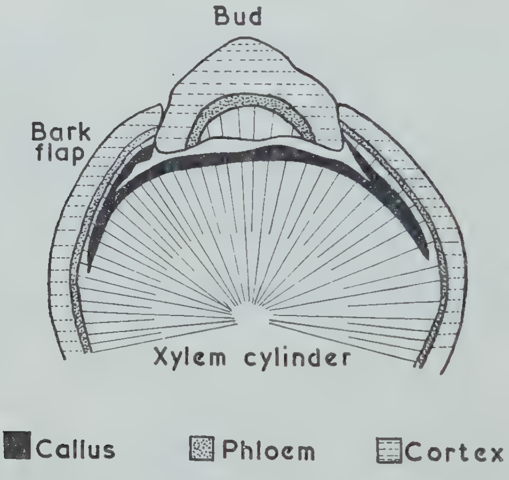
THE DEVELOPMENT OF NEW TISSUES FORMED AS A RESPONSE TO BUDDING

It is necessary to record briefly observations made on the anatomical developments in a young bud-union, since this is the environment in which vascular nodules were found.

When the bark is lifted in the budding operation the break between wood and bark tissue occurs in the undifferentiated xylem, at a point where the vessels are almost fully expanded, but not yet lignified. The whole of the cambial zone remains attached to the inside of the bark flap. Three main phases can be distinguished in the subsequent establishment of the bud.

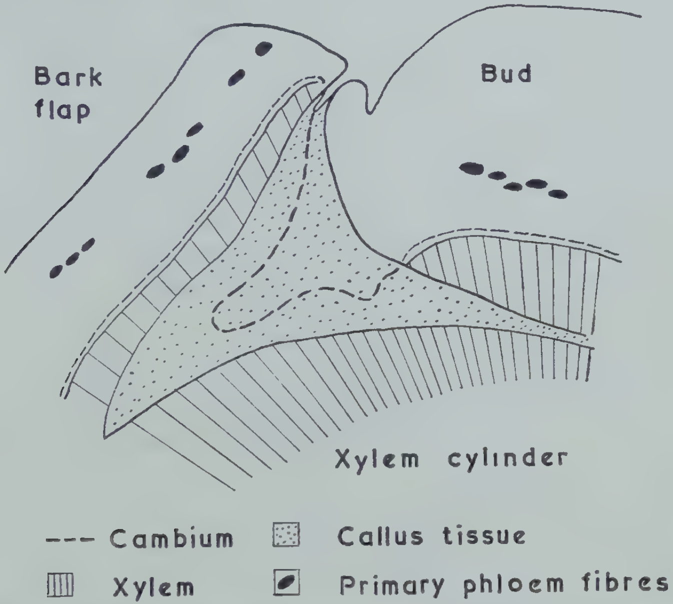
1. The production of callus which surrounds the bud shield and holds it firmly in position.
2. The establishment of a continuous cambium between the bud and the rootstock followed by the resumption of cambial activity in the bud.
3. Lignification of the callus and shortening of the cambial connexion.

Callus originates almost entirely from rootstock tissue, mainly from the exposed surface of the xylem cylinder and to a lesser extent from the inside of the bark flap (Text-fig. 1). Very little is produced from the sides of the bud shield. Callus begins to develop 2 days after budding and continues growing rapidly for 2–3 weeks until all internal air pockets are filled up. At first the cells tend to be in radial rows and show no visible differentiation. After 8 days callus production from the inside of the bark flap ceases, normal activity is resumed in the uninjured cambial zone, and xylem which rapidly begins to lignify is produced towards the inside of the bark flap. The callus layer covering the wood cylinder doubles its radial width during the next week, and individual centres of meristematic activity develop in it. Some of these eventually give rise to amphivasal nodules. A secondary development during this phase is the formation of phellogens sealing off dying tissues at the tip of the bark flap, and small exposed areas of the bud shield. Three to four weeks after budding the base of the bud shield is completely embedded in callus tissue.



TEXT-FIG. 1. Distribution of tissues in a 3-week-old apple bud-union. (T.S.)

Some time during the next 4 weeks cambial continuity between the bud and the bark flap is established. The time at which this occurs is rather variable but the indications are that once this process begins it proceeds very rapidly and almost simultaneously throughout the callus. Invariably the connecting cambium is deeply involuted and follows closely the outline of the woody cylinder and of the bark flap (Text-fig. 2). About this time parts of the callus begin to lignify and isolated tracheary elements appear in the callus farthest away from the xylem cylinder. Once cambial continuity is established the cambium of the bud starts to function, and as new tissues are laid down the bud itself is pushed farther out.



TEXT-FIG. 2. Location of connecting cambium in an 8-week-old apple bud-union. (T.S.)

During the third phase lignification proceeds rather irregularly throughout the callus. Pockets of the connecting cambium, which has never become fully functional, are left behind in the lignifying callus and new, shorter, cambial connexions arise nearer the tip of the bark flap. With one exception lignification of the callus was complete 12 weeks after budding.

THE STRUCTURE, DEVELOPMENT, AND LOCATION OF VASCULAR NODULES

In both years amphivasal (xylem peripheral) and amphicribral (phloem peripheral) nodules were found. Of the 5 amphicribral nodules 4 were located in the bark flap and 1 in the bud shield. Amphivasal nodules invariably occurred in tissue derived from the xylem. They were more numerous, particularly in the rootstock M. VII, where they were found in 12 out of 13 suitable unions. As many as 14 separate nodules were counted in one transverse section. No nodules developed in treatments without a bud, and where plastic buds were substituted all callus-formation was inhibited. It is possible that the plastic material used was unsuitable, and impeded aeration or exerted some toxic effect.

(*a*) *Amphivasal nodules.* In a few instances amphivasal nodules developed in the incompletely differentiated xylem present at the time of bud-insertion, but characteristically they were situated in callus tissue laid down after budding. The first stage in their development is the formation of individual meristematic centres in the xylem-derived callus approximately 2 weeks after budding. Within these centres elongated cells differentiate, with primary pit fields and a thin layer of callose on their end walls (Pl. 1, Fig. 1). A little later, when the circular nature of the new meristem becomes more defined, the central cells become clearly recognizable as sieve tubes. In the 3-week-old longitudinal section shown in Pl. 1, Fig. 2, a sieve plate, prominent callose deposits, and companion cells cut off from the sieve elements can be clearly seen. In Pl. 1, Fig. 3, the central sieve elements, cut transversely, are clearly distinguishable by their thin walls, angular outline, lack of contents, and densely stained companion cells. At this stage a cambial layer develops around the multicellular meristematic centre, usually starting at the side nearest to the xylem cylinder. The beginning of this cambial sheath is shown in Pl. 1, Fig. 2. The vascular nodule now becomes clearly distinguishable as a separate entity in the callus (Pl. 1, Fig. 4), and its approximately circular outline in both transverse and longitudinal sections indicates that it originates as a spherical body. Nodules vary in size. They may arise between rays or occasionally have rays passing through them (Pl. 1, Fig. 5), but invariably the cambial layer forms around a group of cells in the centre of which there are specialized cells with callose deposits. Once the surrounding cambium starts dividing, the callose deposits thicken and assume the characteristic appearance of callose plugs in sieve tubes. No similar callose deposits occur elsewhere in the callus and there is little doubt that the differentiation of isolated sieve elements in the callus provides a focal point around which new vascular cambia develop. The stimuli responsible for the spontaneous differentiation of sieve elements in the xylem-derived callus remain entirely unexplained. Since the cambial zone adheres to the bark flap when the bud is inserted, it is unlikely that the sieve elements are formed from isolated cambial cells left attached to the surface of the woody cylinder.

New vascular nodules arise up to 4 weeks after budding. Once the cambial sheath has developed it becomes active, cutting off xylem elements to the outside, and thin-walled, non-lignified, phloem-like cells to the inside (Pl. 1, Fig. 5). The phloem-like nature of the internal cells is further supported by the presence of rhomboid crystals in some of them. Pl. 2, Fig. 6, shows two typical vascular nodules in a 20-week-old union. Some vascular elements occur around the periphery of the nodule, but they are absent from the more recently formed xylem which consists of fibres, parenchyma, and ray cells only. It is evident that cambial growth in such a nodule, surrounded by rapidly lignifying callus, must eventually stop, and in the older unions small, completely lignified nodules were found. Frequently, however, adjacent nodules coalesce, forming organs of much greater potential vigour which continue to grow for several weeks. In some unions the coalescence of vertically adjacent nodules had effectively resulted in the formation of amphivasal bundles (Pl. 2, Fig. 7), up to 1·5 cm. in length. Even the largest organ of this nature must be of finite growth if it cuts off xylem to the outside. In a few nodules, however, usually those with a vigorous ray passing through them, meristematic activity changes from a concentric to an eccentric type of growth, so that only the cambium on the internal half of the nodule remains active, and eventually the nodule with its phloem directed outwards is pushed through the callus and joins up with the new cambium developing between the bud and the bark flap. This is similar to developments described by Mosse (1953) in ring-grafted apple stems.

(*b*) *Amphicribral nodules.* Invariably amphicribral nodules developed in tissue derived from the phloem. In every instance they were formed in positions where the cambium had died or ceased to function while the young adjacent phloem was still alive and starting to proliferate. Such conditions occurred with some regularity near the tip of the bark flap. Within the proliferating phloem some isolated protoxylem-like elements differentiate, and analogous with the amphivasal nodules, a sheath of cambial cells develops at some distance around this tracheary centre. A young nodule of this sort is shown in Pl. 2, Fig. 8. Some older nodules in which growth from the cambial sheath has already taken place are shown in Pl. 2, Fig. 9. Vessel elements and normal xylem have developed in the centre of the larger nodule. Pl. 2, Fig. 9, also illustrates the characteristic location of these nodules at the tip of the bark flap. The position of the phloem-fibre bundles in the adjacent tissue makes it certain that the nodules originate from proliferating cells of the young secondary phloem. The presence of rhomboid crystals in cells surrounding the nodules supports this interpretation. In the present instance the resumption of meristematic activity in the phloem, the initiation of tracheary centres, and the development of the cambial sheath all occurred within 4 weeks. The method of initiation of such nodules closely resembles that of vascular nodules arising in tissue culture. As with the amphivasal nodules the forces leading to the differentiation of the central protoxylem elements remain unknown, but such development occurred only where there was a region of active phloem tissue which had, for some reason, no xylem counterpart.

DISCUSSION

The resumption of meristematic activity in partially differentiated cells and the subsequent redifferentiation of their derivatives are well-recognized stages in the process of wound healing in higher plants. The anatomical developments leading to the establishment of vascular nodules in apple bud-unions closely follow this well-established pattern. The similarity between the processes leading to the initiation of new meristematic centres in tissue cultures and in bud-unions is very striking in view of the great difference in the environmental conditions under which they arise; in the one case a mass of undifferentiated tissue in a nutrient solution, in the other a virtually intact plant. In both instances the focal point for the development of the cambial sheath is an isolated vascular element, either tracheary or sieve tube, arising in a more or less undifferentiated mass of callus. The interest of the present investigation lies in the simultaneous development of both amphivasal and amphicribral nodules in close proximity in the same plant, and in the clear relationship between the type of nodule formed and the origin of the tissue in which it arises. Amphivasal nodules occurred in xylem-derived callus, amphicribral nodules in proliferating phloem tissue. This regular relationship is supported by various reports of the origin of isolated vascular cambia in wound tissue. It also agrees with Gautheret's observations on tissue cultures, where phloem-derived cultures of carrot tissue gave rise to amphicribral nodules, while amphivasal nodules formed in xylem-derived Jerusalem artichoke cultures. The present evidence strongly supports Gautheret's (1957) view that 'the particular orientation of these structures (vascular nodules) is conditioned by the nature of the pre-existent tissues'. Bloch (1952) regards amphivasal nodules as 'atypical' and speaks of an inversion of cambial activity in them. It seems that the direction of cambial activity in a vascular nodule is controlled by environmental conditions operating when the initials of the cambial sheath are laid down, and that it is determined by the central element, so that this will be surrounded by more tissue of its own kind when the cambium begins to function.

Whereas vascular nodules arise spontaneously in tissue cultures, their initiation depended on the presence of a living bud in the material here described. The evidence is, however, inconclusive since the plastic bud substitutes exerted some deleterious effect on callus formation, and earlier work suggests that vascular nodules can form as a simple wound response. If this is so, the conditions favouring their initiation deserve further study, especially since they may act as root initials (Steward, Mapes, and Mears, 1958), and may therefore be of significance in the rooting of cuttings.

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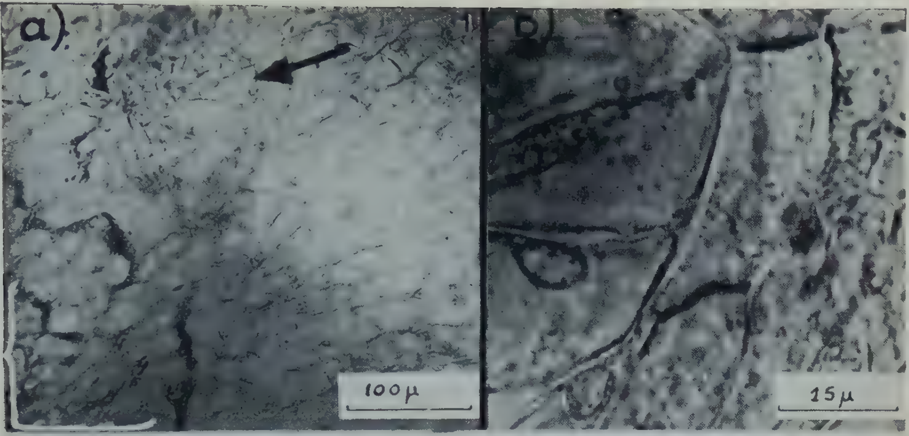

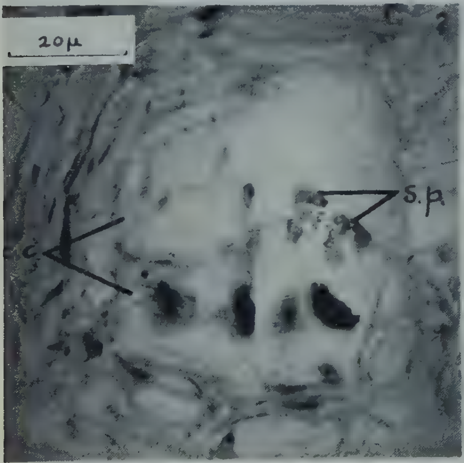

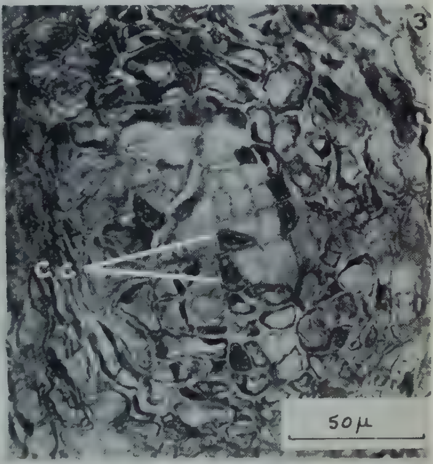

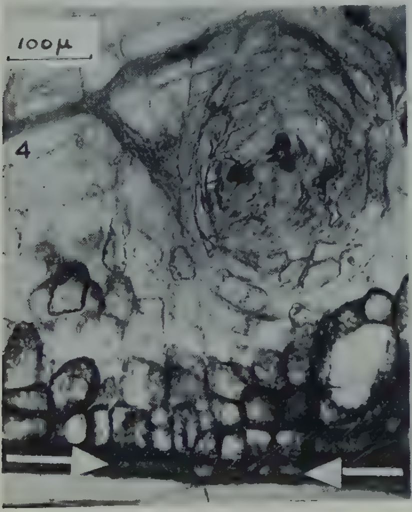

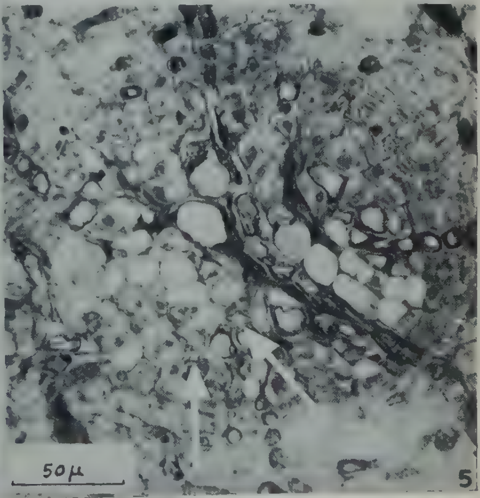

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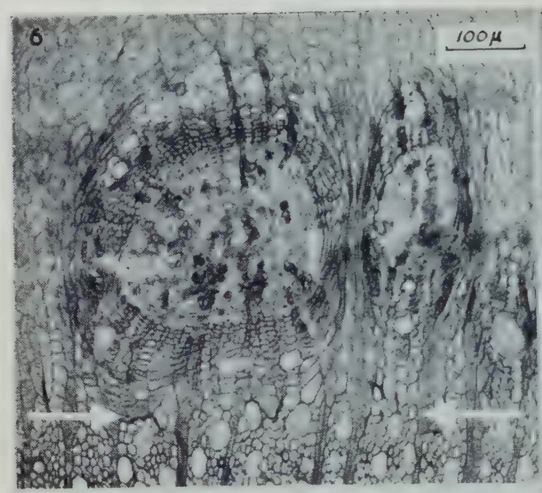

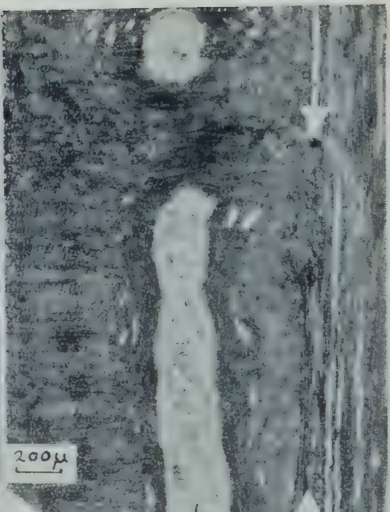

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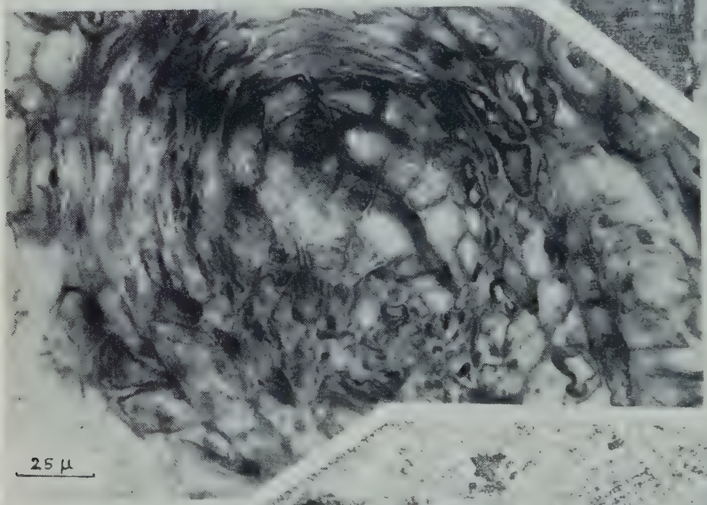

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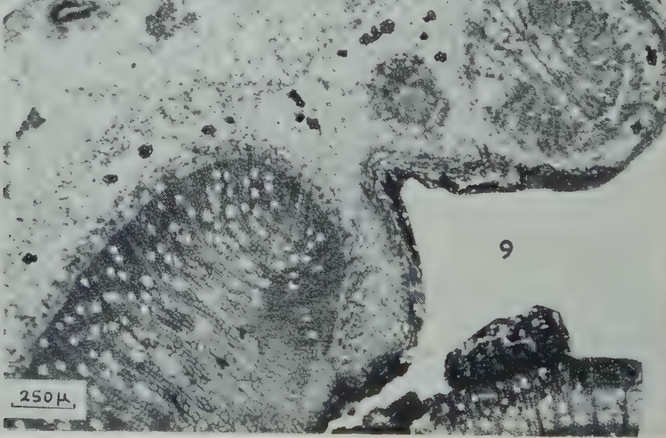

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BARBARA MOSSE and M. V. LABERN

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EXPLANATION OF PLATES

Plate 1

Fig. 1. T.S. through the xylem-derived callus of a 16-day-old bud-union of Fortune/M. VII, showing (*a*) position and (*b*) detailed structure of a developing sieve element. Xylem present at the time of budding is enclosed by brackets.

Fig. 2. L.S. through a very young amphivasal nodule developing in the xylem-derived callus of a 3-week-old bud-union of Fortune/M. VII. Note the central sieve elements with companion cells (c.c.), sieve plates (s.p.), and callose deposits stained with resorcin blue.

Fig. 3. T.S. through a young amphivasal nodule developing in the xylem-derived callus of a 16-day-old bud-union of Fortune/M. VII. Note the central sieve elements with densely stained companion cells (c.c.).

Fig. 4. L.S. through a young amphivasal nodule developing in the xylem-derived callus of a 3-week-old bud-union of Fortune/M. VII. Note the heavy callose deposits in the central cells. White arrows mark the boundary between the original xylem (below) and the callus derived from it (above).

Fig. 5. T.S. through a growing amphivasal nodule in a 4-week-old union of Fortune/M. VII. Note the ray passing through the nodule and the rhomboid crystals indicated by arrows.

Plate 2

Fig. 6. T.S. through two fully developed amphivasal nodules in a 20-week-old union of Fortune/M. VII, showing new vessel elements formed from the nodule. Arrows mark the boundary between the original xylem (below) and the callus (above).

Fig. 7. L.S. through a 12-week-old bud-union of Fortune/M. VII, showing an amphivasal bundle formed by coalescence of longitudinally adjacent nodules. Arrows mark the boundary between the original xylem (right) and the callus derived from it (left).

Fig. 8. T.S. through a young amphicribral nodule formed at the tip of the bark flap in M. II, 8 weeks after budding. Note the central protoxylem-like elements.

Fig. 9. T.S. through the tip of the bark flap of M. II, 12 weeks after budding, showing the structure and position of two amphicribral nodules. The secondary phloem fibres separating this year's from last year's phloem are outlined in ink.

On the Seed *Vesquia tournaisii* C. E. Bertrand, from the Belgian Wealden

BY

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(Imperial College of Science and Technology, London S.W. 7)

With two Figures in the Text

ABSTRACT

This hitherto little-known seed is redescribed from two new specimens. The structure of the stone and internal cutinized membranes is described. The new knowledge tends to confirm the attribution of this seed to the Taxopsida.

INTRODUCTION

C. E. BERTRAND in 1883 proposed the name *Vesquia tournaisii* for some fossil seeds collected from black clays now recognized as part of the Wealden formation near Tournai, Belgium. Although he gave only a brief description without illustrations, it is perfectly clear that Bertrand's seeds were specifically identical with those described here. The two new seeds were collected probably about the beginning of the century by E. de Jaer from the same locality and deposited in the Institut Royal des Sciences Naturelles de Belgique. I have been unable to trace Bertrand's original specimens.

The seeds are preserved as lignite without mineralization. One of the specimens is perhaps slightly compressed, but the other is apparently undistorted. Three methods have been used to study the anatomy: (1) softening of fragments in weak KOH followed by a few minutes in hydrogen peroxide and sodium carbonate, which has the effect of macerating the tissues so that when gentle pressure is applied the cells tend to separate; the eventual dissolution of the cells is prevented by applying a trace of $CaCl_2$; (2) preparation of cuticles by the ordinary method; (3) stripping superficial layers of cells on cellulose acetate film.

DIAGNOSIS

Vesquia C. E. Bertrand. Seed stone spherical but with a conical micropylar beak. Two opposite cavities in the wall of the stone beginning near the base as shallow grooves and extending upwards as canals into the substance of the stone and ending blindly about three-fifths of the distance to the micropyle. Outer surface of the stone smooth, without any distinct basal scar. Wall of the stone thick, becoming thinner upwards, enclosing a spherical or ovoid locule. Inner surface smooth and regular except for two slightly raised areas each bounded by an inverted V-shaped groove and lying directly over the ending of the canal in the wall of the stone. Wall composed of stone cells

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except for a thin layer of elongated cells resembling transfusion cells extending from each V-shaped groove into the wall and joining the outer side of the canal situated behind the groove.

Vesquia tournaisii C. E. Bertrand. Seed stone about 1 cm. in diameter; apical beak about 1·5 mm. long, 0·6 mm. in diameter at its extremity. Wall of stone 2–2·5 mm. thick at the base, diminishing to 0·5 mm. at the base of the micropylar beak, composed of angular stone cells with walls 10–20 μ thick and perforated by abundant forking pit-canals; cells more or less isodiametric (40–70 μ in diameter) through most of the thickness of the wall, but smaller in the innermost layers and somewhat radially elongated in the outermost. A layer of shortly rectangular cells (as seen in surface view) with pitted anticlinal walls 2–4 μ thick present outside the stony layer at least on the apical beak. Surface of the locule, at least near the micropyle, bounded by an epidermis of rectangular cells with anticlinal walls 1·5–2 μ thick. Nucellus cuticle showing clear but thin cell outlines; cells rectangular, 20–30 μ broad, 4–6 times as long as broad. Megaspore membrane only about 0·5 μ thick (as seen in folds), without granulations, showing faint outlines of angular, more or less isodiametric cells.

Horizon: Wealden.
Locality: Tournai, Belgium.
Neotype: Specimen 1 (Fig. 1, A–D).

FURTHER DESCRIPTION

The slightly larger of the two specimens (specimen 2; Fig. 1E) is almost perfectly spherical except for the apical beak, but the other is slightly compressed in a vertical plane at about 30° to the plane in which the canals lie (Fig. 1D). In this specimen also the canals are about twice as broad as in the larger specimen, and the tip of the micropylar beak is notched instead of being obliquely truncate. The notch lies in approximately the same plane as the canals, but it is not certain whether this is a genuine structural feature. The surface of the stone is typically smooth, but bears about nine very shallow and ill-defined longitudinal furrows. The larger specimen shows a number of tiny abraded spots or pits, but these appear to be due only to coarse particles of matrix becoming pressed into the surface. The micropylar beak shows a slightly different surface texture from the rest of the stone, and from this region portions of a cell layer somewhat resembling an epidermis were removed on cellulose acetate film; no trace of such a layer has been found elsewhere on the stone. These cells (Fig. 2D) have rather thick, pitted anticlinal walls, but I have not been able to obtain any evidence that their outer walls bore a cuticle; indeed, the outer walls sometimes appear to be pitted or perforated.

The stone cells are similar to those found in many seeds (Fig. 2B). Their branching pit-canals are a conspicuous feature. Some of the cells have dark reddish-coloured contents.

The most striking feature of the seed is the pair of cavities or canals that run into the substance of the stone. They begin as grooves near the base, and extend upwards on opposite sides to end blindly about three-fifths of the way to the micropyle (Fig. 1F). Bertrand reported finding remains of vascular bundles in the canals, but in my specimens they are quite empty except for particles of matrix. However, each canal is associated apically with a thin layer of tissue which runs through the stone and extends in a hood-like form from the outer edge of the tip of the canal, over the end of the canal, to an inverted V-shaped groove in the side of the locule (Fig. 1 F–I). The cells of this peculiar layer are elongated and often form sinuous rows; some of them show no obvious pits, but others have conspicuous round or elliptical pits

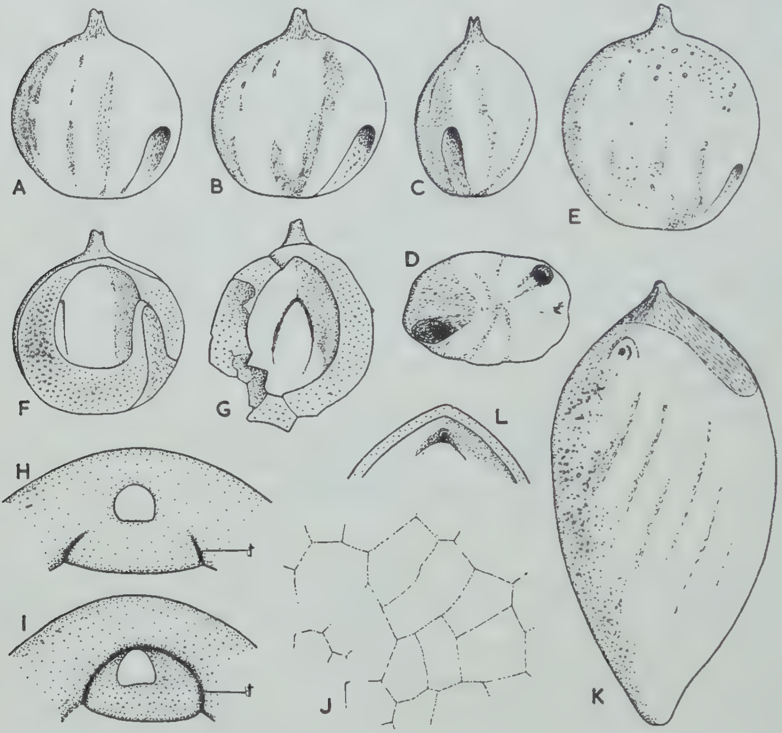

FIG. 1, A–J, *Vesquia tournaisii* C. E. Bertrand. A–D, four views of specimen 1 (Neotype), ×2·4; E, specimen 2, ×2·4; F, specimen 1 broken longitudinally through one of the canals, ×2·4; G, specimen 2 broken longitudinally showing the inverted V-shaped groove on the side of the locule associated with one of the canals, ×2·4; H, I, diagrammatic transverse sections through a canal at two levels, based on dissections of specimen 2, showing the layer of transfusion tissue (*t*) and its association with the canal and the groove, ×8; J, faint cell outlines visible on the megaspore membrane, ×312 (slide M8, specimen 1). K, L, *Torreya nucifera* Sieb. & Zucc. K, stone removed from the aril showing the region at the top covered by an epidermis, and just below, on the left, one of the two foramina, ×2·4; L, part of the stone cut in a tangential longitudinal plane, showing one of the foramina from the inside of the stone, ×2·4.

which sometimes show evidence of a border. These cells resemble transfusion tracheids (Fig. 2E).

Nothing remains of the seed contents except fragments of cutinized parts. The larger specimen had already been broken open and repaired before it came into my hands, and no loose fragments remained inside. A fragment of an epidermal layer, however, was removed on cellulose acetate film from just below the micropyle in this specimen; a typical portion of this is illustrated in Fig. 2C. Amongst the debris in the other (smaller) specimen were recognized fragments of two different kinds of cutinized epidermis, a small cap-like structure and also fragments of another cutinized membrane assumed to be the megaspore membrane. One of the two kinds of epidermis fragments was exactly similar to the piece removed from the inner surface of the stone of the other seed. This therefore represents the inner epidermis of the integument

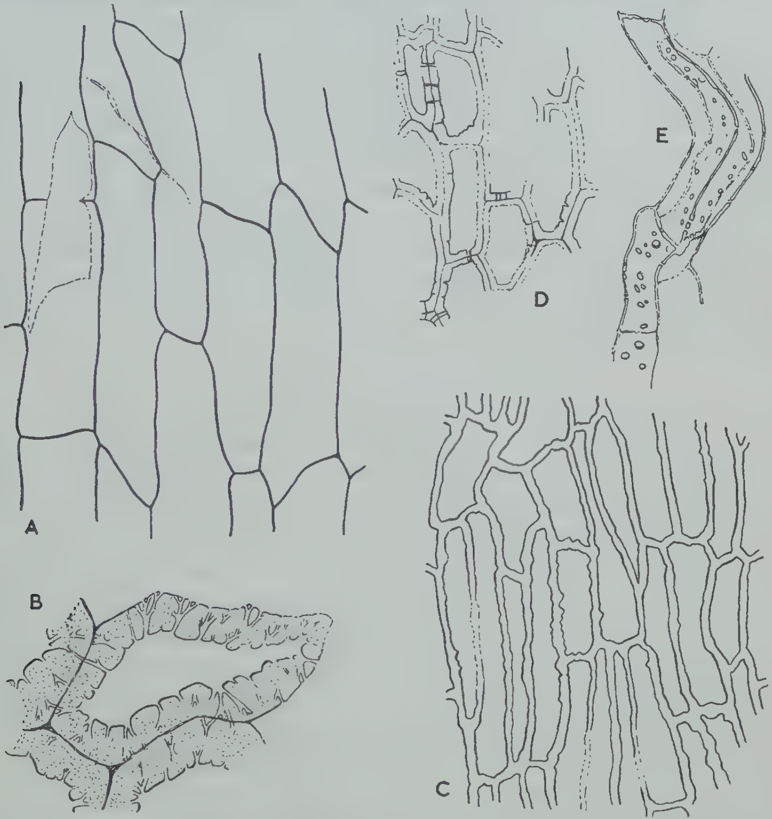

FIG. 2. *Vesquia tournaisii* C. E. Bertrand. A, cell outlines from the nucellus cuticle (slide M12, specimen 1); B, stone cells in optical section from a macerated fragment (slide M4, specimen 2); C, epidermal cells from the inside of the integument (slide M5, specimen 2); D, cells removed from the outer surface of the micropylar beak (slide M11, specimen 1); E, pitted cells from the transfusion layer in the stone (slide M7, specimen 2). All ×312.

which must have somehow separated from the stone. In places, the remains of a sub-epidermal cell layer were visible on this epidermis, but were not sufficiently well preserved to allow description. The cuticle of this epidermis would withstand only mild maceration. The second kind of epidermis in the debris had a rather tough although thin cuticle which withstood maceration in Schultz's fluid and showed thin but very clear cell outlines (Fig. 2A). The small cap (about 1 mm. in diameter), upon maceration, revealed similar cells, especially towards the frayed edges, but was thick and dark with no clear cell markings at the centre. The cap, together with the fragments showing similar cell outlines, was taken to represent the nucellus epidermis. Judging by the total amount of epidermal fragments of the two kinds, I estimate that the nucellus and integument must have been free to perhaps one-third to one-half of the way down the seed. Fragments of another cutinized membrane which withstood maceration were abundant in the debris. This was a clear, very thin membrane showing faint outlines of angular, more or less isodiametric cells. This is believed to represent the megaspore membrane, and the cells, the outermost layer of the female gametophyte.

DISCUSSION

The absence of a basal scar and the fully exposed openings of the canals leading into the stone, both suggest that the seed was originally surrounded by some fleshy layer united to the stone except possibly at the top, where there is some indication that an epidermis may have been present. The flesh could have been part of the integument as in many gymnosperms, an aril as in *Torreya* or a series of scales as in *Juniperus*.

The broad, blindly ending canals somewhat resemble the resin cavities present in the stone in many species of *Juniperus* and certain other conifers. However, they contain no resinous remains. The presence of the associated layer of cells resembling transfusion tissue suggests that Bertrand was right in regarding the canals as passages carrying vascular bundles. They are too broad to have been occupied completely by vascular tissue, so perhaps the bundles were surrounded by delicate parenchyma and became obliterated together with this tissue and that of the postulated outer flesh. Alternatively, the bundles could have been pulled out if the stone eventually separated from the flesh naturally.

Although *Vesquia* is distinctly gymnospermous, its peculiar structural features make it stand apart from most known living and fossil gymnosperms. The seed stones of *Ginkgo* and cycads are comparable in size; but in *Ginkgo* the stone is ridged, and in cycads there is always a basal scar representing the point of entry of the main vascular supply to the nucellus. Moreover, in neither is there a prominent micropylar beak, and in both the megaspore membrane is thick and distinctly granular.

In certain features *Vesquia* may be compared with the seeds of species of *Juniperus*, particularly species in which the fruit contains only a single seed.

However, the resin cavities often present in the stone appear to be fundamentally different from the canals in *Vesquia*, and there is never such a prominent micropylar beak. Further, although the stone is often of comparable thickness, the stone cells in at least two species (viz. *J. communis* L. and *J. virginiana* L.) are different in being relatively thicker-walled and in having usually unbranched pit-canals. The megaspore membrane in *Juniperus*, although thin, is distinctly granular.

Bertrand compared *Vesquia* with the taxads (including *Phyllocladus* and *Cephalotaxus* which were then classified in the same group). The extremely thin stone without a vascular supply in *Phyllocladus* is clearly quite different. *Cephalotaxus* has a two-ribbed, smooth stone with nothing that compares with the canals in *Vesquia*. The resemblance to the true taxads as now understood (Florin, 1948) is, I believe, fundamental, although in no living taxad is the seed stone very similar in general appearance. *Taxus* has a small stone covered almost completely by a rather horny epidermis, and at the base the hilum shows two tiny scars which represent vestigial vascular strands. *Austrotaxus* Compton and *Nothotaxus* Florin (=*Pseudotaxus* Cheng) have similar seeds, except that in the former the vascular system is apparently better developed (Saxton, 1934). *Torreya* appears to be much more like the fossil. It has a large, although rather thin-walled stone, covered by an epidermis only at the top where it is free from the aril (Fig. 1K). There is a micropylar beak, and a short distance below on either side there is a scar (foramen) which represents the place of entry of a vascular strand. In the young ovule, according to Oliver (1903), incipient vascular strands run from the foramina round the integument at the base of the free part of the nucellus. In the mature seed, the foramina are marked on the inside of the stone by broad, inverted V-shaped scars (Fig. 1L), and from these points transfusion tracheids radiate into the inner soft tissue of the integument. This peculiar feature seems to represent an important similarity to *Vesquia* in spite of the obvious differences in shape and proportions and the absence in *Torreya* of broad canals leading into the stone. The stone cells in *Torreya* (and also in *Taxus*) are of the same kind as in the fossil. There is some evidence that in *Vesquia* the nucellus and integument were less fused than in *Torreya*, where these parts become free only at about the level of the foramina. As the foramina in *Vesquia* are situated lower down the stone, it may well be that here too the nucellus became free at about this level.

I have examined apparently fully grown seeds of *Amentotaxus* Pilger in herbarium material. As Florin (1948) pointed out, this genus resembles *Torreya* in the extensive intercalary growth of the seed, so that the aril is fused to the stone except at the top. No vascular bundles enter the integument, so the stone lacks foramina. The stone is very thin.

My attempts to demonstrate a megaspore membrane in *Torreya* failed. In *Taxus* and *Amentotaxus*, however, there is a membrane round the gametophyte which compares well with the supposed megaspore membrane in *Vesquia*, except that the outlines of the outermost cells of the gametophyte are clearer. Unlike the membrane of most gymnosperms, it is not granular.

Amongst the better-known fossil seeds there are few that are comparable with *Vesquia*. The form-genus *Allicospermum* Harris which includes seeds of the *Ginkgo* and cycad type would probably also include *Vesquia* if specimens were preserved so as to obliterate the canals and associated structural features; but no *Allicospermum* is known with a similar megaspore membrane.

The seed of *Stachyotaxus*, a member of a group of Mesozoic conifers now recognized as a distinct family, Palissyaceae Florin (1958), has been described by Harris (1926). It was an orthotropous seed borne in an open, asymmetrical aril, and had a thick stone. In details, however, it differed from *Vesquia*, for the nucellus was free to the base, the integument cuticle (both inside and outside) showed polygonal, not elongated cells, and the megaspore membrane was granular. Moreover, the Palissyaceae are unknown in the later Mesozoic.

Cephalotaxospermum Berry (1910), based on a species from the Cretaceous of North America, in general shape and size is comparable with *Vesquia*, but nothing is known of its detailed structure. This seed was found associated with vegetative remains described as *Tumion* [=*Torreya*] *carolinianum* Berry (1908), but it is not clear whether this plant resembles *Torreya* in its detailed epidermal structure.

Although vegetative remains of taxads are of fairly frequent occurrence in the Jurassic and Cretaceous, and one or two examples of apparently immature ovule-bearing organs have been recorded, as far as I am aware no mature seeds have been described. Largely on the evidence of critically studied vegetative material (with cuticles), both *Taxus* and *Torreya* are known to be represented in the Jurassic of north-west Europe (Florin, 1958). These genera have also been reported from the Jurassic and Cretaceous in other parts of the world, but identification has usually been based only on general shoot morphology; in all probability, however, they were widely distributed. There is now good evidence from well-preserved vegetative material that other, now extinct genera of taxads were present in the Mesozoic (Florin, 1958): thus, *Palaeotaxus* Nathorst (L. Jurassic, Sweden), *Storgaadia* Harris (L. Jurassic, E. Greenland), *Bartolinodendron* Florin (Jurassic, Denmark), *Tomharrisia* Florin and *Marskea* Florin (M. Jurassic, Yorkshire), and *Cephalotaxopsis* Fontaine (L. Cretaceous, Virginia) all show affinity with the taxads. It is not unlikely that some of these extinct genera may have had seeds differing appreciably from the types exhibited by the living taxads. Moreover, as *Taxus* and *Torreya* are themselves ancient and at the same time present important differences in their seeds, other, now extinct taxads coming from the common ancestral stock may well have had seeds of a somewhat intermediate type. *Vesquia*, in at least one character, namely, the position of the points of entry of the vascular supply, could be regarded as presenting an intermediate condition.

SUMMARY

1. The main distinguishing features of *Vesquia tournaisii* C. E. Bertrand

are: (*a*) the very thick stone with a prominent micropylar beak; (*b*) an opposite pair of canals running up into the wall of the stone from near the base and ending blindly; (*c*) the layer of cells, apparently including transfusion tracheids, extending from near the tip of each canal to the seed-cavity.

2. The seed is distinctly gymnospermous. It may be compared morphologically with the seed of *Torreya*. The megaspore membrane, which is unlike that of most gymnosperms in showing angular cell outlines and in lacking granulations, is comparable with that of *Taxus* and *Amentotaxus*. The fossil is believed to represent an extinct genus of taxads.

ACKNOWLEDGEMENTS

I am indebted to the Director of the Institut Royal des Sciences Naturelles de Belgique for the loan of the specimens, and to the Director of the Museum National d'Histoire Naturelle, Paris, for the loan of herbarium material. I should also like to thank Mr. J. Lewis for showing me seeds of various recent gymnosperms in the British Museum (Natural History), and Miss M. E. J. Chandler for examining one of the fossil seeds.

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Virus-induced Abnormalities of Mitosis in Certain Solanaceous Species

BY

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ABSTRACT

Mitotic abnormalities were observed in a range of Solanaceous species infected respectively with tobacco mosaic virus and aspermy virus. These abnormalities ranged from persistence of the nucleolar material as far as early anaphase, in all cases observed, to a complete amitosis encountered in stem-enations of one species examined, viz. *Petunia violacea*. These findings are thought to support the view that virus particles, multiplying within dividing cells, compete with the nuclear-DNA for the supply of RNA located in the nucleolus.

INTRODUCTION

THOUGH the literature concerning the nature and behaviour of plant viruses is now very large, and a number of inquiries have been directed towards the elucidation of the extra-nuclear cytology of plants infected with virus, very few reports have as yet appeared in connexion with the possible effects of virus upon nuclear behaviour. Thus, an incidental reference by Kostoff (1933), discussed and amplified by Caldwell (1952), established that in *Nicotiana tabaccum* and *Lycopersicum esculentum* a proportion of the first meiotic divisions may be arrested in the presence of virus; and Sheffield (1941) reported the stimulation of the nuclei of mature cells in the presence of severe etch virus, resulting in a binucleate condition. In *Vicia faba* infected with bean mosaic virus, Coutinho (1940) noted chromosome fragments and 'strange lateral bridges' in somatic metaphases and anaphases seen in root tips, and (1942) suggested the existence of a natural competition between genes and viruses present in the same cell for the materials necessary for multiplication. This author assumed genes and viruses to have 'identic characteristics and behaviour'. Unaware of this work, the present writer (1952) described abnormalities of meiotic behaviour in *Nicotiana glutinosa* inoculated with aspermy virus of tomato, and in a preliminary report (1953) described mitotic abnormalities in root tips of tomato plants inoculated with tobacco mosaic virus. The view was tentatively advanced that, in the presence of virus, RNA is withheld from the dividing chromosomes and remains within the nucleolus, the normal disintegration of which is arrested during prophase.

Several Solanaceous species have been subjected to more extensive investigation in an endeavour to substantiate these findings, and the results obtained with certain species of *Nicotiana*, *Solanum*, *Hyoscyamus*, *Physalis*, *Lycopersicum*, and *Petunia* are discussed in this report.

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MATERIALS AND METHODS

The viruses employed were aspermy of tomato and the aucuba strain of tobacco mosaic virus. Cuttings of extensively diseased shoots of the various species and varieties under investigation were rooted without difficulty in water. Control cuttings of disease-free plants were similarly rooted. Extracts from samples of crushed roots of all the species investigated were tested for the presence or absence of virus.

The possibility of using 'squash' preparations was investigated, but these methods proved less suitable for the purposes of this type of inquiry than those obtained by the traditional embedding and sectioning technique. The special advantages of smear procedures in general is not here questioned, but the writer's personal experience of these, as employed in cytotaxonomic studies of a range of *Salix* species (Wilkinson, 1944) and also of certain *Cymbidium* varieties (unpublished), has been that the considerable pressures involved in these methods result in a general distortion of nuclear structures, and, in particular, a tendency for chromosomes to fragment. In a study of the present type it is obviously very important to avoid errors arising from unsuitable technique.

A Feulgen–May–Grunwald staining procedure was employed for the simultaneous display of DNA- and RNA-containing structures, and nucleolar bodies were then stained bright blue. When May–Grunwald staining was used in conjunction with the standard iron–alum haematoxylin procedure, nucleolar structures appeared as dark blue-grey objects and were easier to photograph.

OBSERVATIONS

Confirmation of the presence of virus in roots arising adventitiously from diseased cuttings was obtained by inoculating control plants with extracts from crushed roots. It is of interest to note that evidence was frequently obtained that the virus is concentrated in the root-tip of such adventitious roots. Thus, in a typical case, lesions were produced upon *Nicotiana glutinosa* as a result of inoculating with root extracts from (*a*) about 6 mm. of root including the tip, and (*b*) the next 18–20 mm. of root behind the tip of tomato plants infected with a.m.v. In (*a*), 21 lesions (8–7–3–3) appeared on four leaves, whilst in (*b*), a total of only 4 lesions (2–1–1–0) resulted.

Abnormalities of mitosis, such as have previously been reported for certain species (Wilkinson, 1953), were found to occur in varying degree in the region of elongation behind the meristematic apex of the root in all the Solanaceous species studied. In nearly all cases the first indications of abnormality appeared at prometaphase, during which in healthy cells the fully contracted chromatids converge towards the equatorial plane of the spindle, accompanied simultaneously by dispersal of the nucleolar material. Of a minimum of 200 mitoses observed for each species in growing tissues of diseased specimens, 5–15 per cent. exhibited a persistence of nucleolar structures at least as far as the commencement of anaphase (Table 1). The nucleolus did not retain its spherical form characteristic of early prophase, but presented a lobed and distorted appearance, often extending as a finger-like vesicle across the equatorial plane of the spindle. Thus, polar views of the metaphase chromosomes sometimes included a nucleolar remnant of diameter considerably less than that of the interphase nucleolus. In addition to this observation in Solanaceous species, it is of interest to note in passing that the writer has detected similar abnormalities for the Orchidaceae, in certain cultivars of *Cymbidium*, heavily infected with a virus which is well known to growers as invariably being present in some strains, such as 'Alexander Westonbirt'.

TABLE 1

The Percentage of Abnormal Mitoses Observed in Solanaceous Tissues

Species	Virus	Nucleolar vesicles	Anaphase bridges	Giant nuclei	Amitosis
Lycopersicum esculentum	aspermy	15	7	—	—
,, ,,	a.m.v.	13	6	—	—
Nicotiana glutinosa	aspermy	14	6	—	—
,, ,,	a.m.v.	13	4	—	—
,, *sylvestris*	a.m.v.	12	5	—	—
,, *rustica*	a.m.v.	14	6	11	—
Solanum nodiflorum	aspermy	15	7	—	—
,, ,,	a.m.v.	14	7	—	—
Hyoscyamus niger	aspermy	8	3	—	—
Physalis alkekengi	aspermy	5	2	—	—
Petunia violacea, roots	aspermy	6	2	—	—
,, ,, stem enations	aspermy	—	—	—	all amitotic

At anaphase, the occurrence of 'thin' chromosomes and the formation of chromatin bridges were noted as an abnormality, and sometimes spindle breakdown resulted in either a mere scattering of the chromosomes, or else failure of the disjoining chromosome groups to separate, with the consequent production of giant nuclei.

The most remarkable abnormality was, however, seen in *Petunia* (var. 'Fire Chief') in certain stem-enations which developed with great rapidity from mid-December until the beginning of February. In these examples, rapid cell-multiplication led to the production of extensive flap-like ridges upon young stems, and it was to be expected that numerous mitotic figures would be found in sections prepared for cytological observation. Careful examination revealed no such mitoses, but many examples were encountered of elongated and constricted nuclei which appeared to be undergoing an atypical form of mitosis. Sections of young enations stained with May–Grunwald exhibited a marked and evenly distributed concentration of RNA which differentiated them sharply from the cortical tissue of the stems from which they arose.

DISCUSSION

Such abnormalities of nucleolar structure and behaviour as probably occurred were unmentioned in Kostoff's report upon *Nicotiana* species, published in 1933; this is, perhaps, not surprising, for at that time nucleolar structures, though known to occur with great constancy of form in nearly all interphase nuclei, were not widely regarded as possessing any functional significance. Moreover, most cytologists were at that time preoccupied with the mechanics of chromosome behaviour; Heitz (1931) had only recently demonstrated the relationship between the nucleolus and the satellited chromosomes, and nearly a decade was to elapse before a plausible role could be established for the nucleolus as a repository of ribonucleic acid (Caspersson and Schultz, 1940), a substance now thought to play a far larger part in cell metabolism than was formerly supposed. It is only in comparatively recent years that an internal differentiation of nucleolar substance into a filamentous organoid, the nucleolonema, has been demonstrated (Estable and Sotelo, 1951), together with the elucidation of a nucleolonematal cycle in a few cases. In *Nicotiana glutinosa* infected with aspermy virus the present writer reported the characteristic subdivision of the nucleolus, initially present as a single spherical body, into a number of smaller spheres which remained attached to the collapsed threads during the first meiotic prophase in abnormal pollen mother-cells.

In the majority of the very large number of organisms in which the mitotic cycle has been observed in detail, the nucleolar material disperses before the end of prophase, and reassembles during telophase, preserving a sequence of morphological changes which are characteristic of the species and which may, indeed, be employed as taxonomic criteria (Gates, 1942), especially in connexion with the degrees of polyploidy (Wilkinson, 1944). In normal cell-division, the nucleolar material, and especially its RNA component, is widely thought to play a leading role in the organization of the chromosomes, possibly by processes involving 'template' mechanisms. Any failure or partial failure of the nucleolar RNA to disperse during prophase may reasonably be expected to be followed by varying degrees of disruption in the subsequent stages of mitosis. Thus, the widespread occurrence of the suspension of the dispersal of nucleolar material in dividing cells containing virus, as reported here for Solanaceous species, may well have profound significance. It is of interest to note that Coutinho (1942, fig. 6) includes a photograph of a late metaphase stage in a root of *Vicia faba* infected with bean mosaic virus, in which persistent nucleolar material is clearly visible, though he draws no attention to this in his account.

No obvious morphological abnormalities of the metaphase chromosomes have so far been detected in Solanaceous species infected with virus. Coutinho (1942) reported lacunae in certain pairs of chromosomes at metaphase for *V. faba*, and claimed that these were correlated with genetic deficiency.

At anaphase, the clear chromatin bridges detected for the wide range of species shown in Table 1 provide evidence of a localized lack of 'pellicle', or nucleolonematal, material; this is doubtless a consequence of the fact that the chromosomes at late prophase were deprived of a part of their nucleolar substance. A more extreme abnormality sometimes seen at anaphase was spindle-breakdown, especially in *Nicotiana glutinosa* and *N. rustica*. This resulted in either the scattering of chromosomes or the formation of polyploid nuclei. It is of particular interest as Brachet (1957) has confirmed an earlier view of Kaufmann and Das that RNA is necessary to the formation and functioning of the spindle.

The widespread occurrence of amitotic divisions in rapidly growing stem-enations in *Petunia* affords the most striking instance of abnormality so far found. In these, marked elongation of each nucleus is followed by septation and cleavage into two daughter-nuclei. As is well known, amitotic divisions occur in nutritive structures, such as tapetal cells; they have also frequently been observed in cells which are ageing or degenerating, as in tumours (Drew, 1923). The amitotic mode of division, involving as it does neither the formation of chromosomes nor that of an achromatic figure, is clearly an economical one in terms of nucleolar substance, which would obviously be available in maximum amount for the multiplication of virus particles. Again, since RNA is necessary to the formation of a spindle, a wholly amitotic method of division may well be an extreme type of reaction on the part of the nucleus to an acute shortage of that substance.

Evidence from a number of important investigations carried out during recent years, admirably summarized in Brachet's book, *Biochemical Cytology* (1957), leaves little reason to question the fundamental importance of RNA in the synthesis of plant-virus particles and as a genetic determiner thereof. As Gottschalk writes (1957): 'From recent data on bacterial viruses and on tobacco mosaic virus, it seems most likely that virus nucleic acid . . . is the main, if not the only, virus component responsible for the diversion of the host cell metabolism from the synthesis of host protein and host nucleic acid to the synthesis of the corresponding virus materials. This redirection, as any redirection of a metabolic pathway, can only be visualized as the result of competition.' It is the present writer's opinion that in plant cells undergoing division and containing virus particles, this competition between such particles and the nuclear DNA for the RNA-content of the nucleolus is the cause of such abnormalities of mitosis as may occur; and one possible path for the dissemination of virus is obviously via such abnormally dividing cells.

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